Spinal cord cell culture: A model for neuronal development and disease

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SPINAL CORD CELL CULTURE : A MODEL FOR NEURONAL DEVELOPMENT AND DISEASE

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TO MARIANNE,

FOR HER UNERRING PATIENCE,

ENCOURAGEMENT AND SUPPORT.

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SUMMARY

Conditions have been developed for the successful culture, in serum-free medium, of neurone-rich, foetal rat spinal cord cells for two purposes. These were, firstly, for characterisation of muscle-derived neuronotrophic factors and secondly, for analysis of monoclonal anti-neuronal antibodies.

Human skeletal muscle extracts were shown to increase levels of neurofilament, overall protein and choline acetyltransferase activity in the cultured cells and also to extend considerably their survival.

A number of monoclonal antibodies with specificity for neural tissue antigens have been generated and some have been characterised. In particular, an anti-neurofilament antibody (2F7) has been used routinely for identification and assay of cultured neurones, while a second antibody (3C4a) appears to recognise a serum-inducible neuronal surface glycoprotein.

Conditions have also been developed for the successful growth and maintenance of foetal human spinal cord neurones in both serum-supplemented and serum-free media. These cultures have been used in comparative studies.

The rat spinal cord cell cultures have also been used in studies of Motor Neurone Disease (MND). Binding of MND serum immunoglobulins to the cultures has been examined by using immunofluorescence and ELISA techniques and sera have been tested for neurocytotoxic activity.

LIST OF ABBREVIATIONS

(Common chemical symbols not included).

ACh	Acetylcholine
ACHC	Aminocyclohexane carboxylic acid
AChr	Acetylcholine receptor
AEC	3-amino 9 ethylcarbazole
AIDS	Acquired immunodeficiency syndrome
ALS	Amyotrophic lateral sclerosis
ANF	Anti-neurofilament
BDNF	Brain derived neurotrophic factor
α-Bgt	α-bungarotoxin
(L)-BMAA	β- N-methylamino-L-alanine
BME	Basal medium (Eagles)
(L)-BOAA	β-N-oxalylamino - L-alanine
BSA	Bovine serum albumin
BSP-1/2	Brain specific protein (Antigen)
BSS	Balanced salt solution
C-CTF	Central cholinergic trophic factor
CEE	Chick embryo extract
CG	Ciliary ganglion
CGRP	Calcitonin gene-related peptide
ChAT	Choline acetyltransferase
CIPE	Choroid, iris-ciliary body, and adherent pigment epithelium
ClACh	Chloroacetylcholine
CM	"Cloning" medium
CNS	Central nervous system
CNTF	Ciliary neuronotrophic factor
CoA	Coenzyme A

ABBREVIATIONS(continued)

CSF	Cerebrospinal fluid
Da	Daltons
DFP	Di-isopropylfluoro-phosphate
DFU	Deoxyfluorouridine
DHS	Donor horse serum
DMEM	Dulbecco's modification of Eagles' medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNAase	Deoxyribonuclease
DRG	Dorsal root ganglion (ganglia)
EBM	Eagles basal medium
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FCS	Foetal calf serum
FDGF	Fibroblast derived growth factor
FdU	Fluorodeoxyuridine
FGF	Fibroblast growth factor
FITC	Fluoroscein isothiocyanate
FSA	Fibroblast surface antigen
GABA	Gamma aminobutyric acid
GAD	Glutamic acid decarboxylase
GC	Galactocerebroside
GFAP	Glial fibrillary acidic protein
GGF	Glial growth factor
GPI	Glucophosphoisomerase
HAc	Acetic acid (glacial)

ABBREVIATIONS (continued)

HAT	Hypoxanthine, aminopterin, thymidine
HBSS	Hanks balanced salt solution
H-EBM	HEPES - buffered Eagles basal medium
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HI	Heat inactivated
HIV (-1)	Human immunodeficiency virus
HLA	Major histocompatibility complex in man
HM-A	Human medium-A
HP	High power
HRP	Horse-radish peroxidase
HS	Horse serum
HT	Hypoxanthine, thymidine
Ig	Immunoglobulin
KDa	Kilodaltons
LCF	Large cell fraction
LDH	Lactate dehydrogenase
LETS	Large external transformation sensitive (glycoprotein)
LMC	Lateral motor column
LMN	Lower motor neurone
LP	Low power
MAO	Monoamine oxidase
MEM	Minimum essential medium
MND	Motor Neurone Disease
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1,2,5,6-methylphenyltetrahydropyridine
MY-FCS	Myoclone foetal calf serum
N-CAM	Neural-cell adhesion molecule

ABBREVIATIONS (continued)

NGF	Nerve growth factor
NGS	Normal goat serum
NILE	NGF-inducible large external (glycoprotein)
NLK	Neuroleukin
NOPA	Neurite outgrowth promoting activity
NOPF	Neurite outgrowth promoting factor
NRS	Normal rabbit serum
NS-1/2/4	Nervous system (specific antigens)
NSE	Neuron-specific enolase
O.D.	Optical density
OND	Other neurological disease(s)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
P-D	Parkinsonian dementia
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
PEN/STREP	Penicillin-streptomycin
PGF	Polypeptide growth factor
PMA	Progressive muscular atrophy
PNPF	Poly-ornithine binding neurite-promoting factor
PNS	Peripheral nervous system
PORN	Poly-ornithine
PTFE	Poly tetrafluoroethylene
RAN-1/2	Rat anti-neural (antigens)
RNA	Ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute
SCG	Superior cervical ganglion

ABBREVIATIONS (continued)

SDS	Sodium dodecyl sulphate
SFM	Serum-free medium
SLE	Systemic lupus erythematosus
SMA	Spinal muscular atrophy
SSEA-1/3/4	Stage-specific embryonic antigens
SSM	Serum-supplemented medium
TBS	Tris buffered saline
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone (Thyrotropin)
U	Uridine
UMN	Upper motor neurone
UV	Ultra-violet
WGA	Wheat germ agglutinin
X-63	X-63 8Ag Myeloma cell line

PUBLICATIONS

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1. INTRODUCTION

1.1 DEVELOPMENT OF NEURAL TISSUE CULTURE SYSTEMS

1.1.1 SELECTION OF APPROPRIATE SYSTEM

Harrison (1908, 1910) initiated the study of neural tissue in culture with explants of frog spinal cord suspended in clotted lymph. He was successful in answering the question as to the origin of the "long fibres" of the nervous system by observing the outgrowth of nerve axons from cell bodies into the surrounding lymph clot. He also noted that the growing tip of the axon was expanded to form the appropriately named "growth cone", previously described by Cajal (1890) in sections of the developing chick nervous system (see Letourneau, 1983).

The range of neural tissue culture systems has since expanded (rev. Nelson, 1975) to include organ culture, explants, dissociated tissue and neuronal cell lines.

Organ culture involves culture of whole isolated spinal cord usually from frog or chick (Katz & Miledi, 1963).

Explants have been prepared from isolated chick or mammalian sensory and autonomic ganglia (Sobkowicz et al., 1973), from sections of spinal cord (Peterson et al., 1965) and from brain (Pomerat & Costero, 1956; Hild, 1966).

Dissociated tissue cultures normally involve a single cell suspension produced by a combination of enzymic and mechanical procedures. Dissociated cells

are plated out at specific densities (rev. Ransom & Barker, 1981). Re-aggregation of some of the cells may occur spontaneously, or this may be actively encouraged (Seeds & Vatter, 1971). For some purposes, however, re-aggregation of the cells is undesirable and various attempts have been made to reduce it, usually by modifying the substratum (Section 1.1.5).

Dissociated neural cultures provide maximum accessibility for morphological examination. Their large surface area allows for efficient exchange of nutrients and waste with the media. In addition, potential neurotoxic or neurotrophic substances included in the media are in rapid, direct contact with the cells.

Dissociated spinal cord cell culture was initially developed by using embryonic chick as the source (Fischbach, 1972). The neurons had a survival time of only a few days in culture. With foetal mice as the tissue source (Peacock et al., 1973; Ransom et al., 1977), longer term cultures of 3 to 8 weeks duration were reported. A vital factor was the age "in utero" of the foetuses which would only yield "good, healthy cultures" between days 12-14.

Dissociated foetal rat spinal cord cultures (Digby et al., 1985) are prepared in a similar manner to those of mouse, with day 15 foetuses yielding the best cultures irrespective of which medium or substratum is used (Section 2.3.1.6).

Neuronal cell lines (see Nelson, 1977, for review) have been derived either from spontaneously arising tumours of the nervous system (eg. the C-1300 mouse

neuroblastoma which arose in 1940 and was carried by serial passage in mice until adapted to tissue culture by Augusti-Tocco & Sato (1969) or by chemical induction (eg. Schubert & Jacob, 1970). Their properties have been extensively studied (eg. Prasad et al., 1973; 1975; 1976) including their ability to form functional synapses with primary muscle cell cultures (Nelson, 1977).

Each type of system described above has its own advantages and disadvantages and these are summarised in Table 1 (p. 4; from Nelson, 1975). Survival time of the culture may be an important factor in the choice of an appropriate system, and hence this has been added to Nelson's original table.

Table 1 Properties of tissue culture systems (from Nelson, 1975).

Scale graded between minimum (0) and maximum (++++) degrees.

Type of preparation	Distinctive Preparative Procedure	Level of tissue organisation	Reproducibility	Accessibility	Genetic Manipu-lation	Survival Time
Intact system	0	++++	++++	+	+	++++
Organ culture and explants	remove organismic influences	++++ +++	++++ +++	++ ++	+ +	+ ++
Reaggregates & cell culture	disrupt inter-cellular relationships	++ +	++ +	+++ ++++	+ +	+++ ++++
Cell lines	lose growth control	0	++++	++++	+++	++++

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1.1.2 CULTURE OF HUMAN NEURAL TISSUE

Early attempts at the culture of human neural tissue were restricted to explants. Peterson et al. (1965), for example, successfully grew a spinal cord explant from a 6 week old human embryo. Explants were not successful when prepared from 12-16 week foetuses.

The first report of the successful culture of dissociated human neurones was by Scott (1971), who used dorsal root ganglia (DRG), from 10-16 week foetuses, grown in serum supplemented medium (SSM) containing a high potassium ion (K^+) concentration. The neurones remained viable for up to 11 weeks. Proliferation of the connective tissue cells was observed to be much slower than in the comparable chick system. Kennedy et al., (1980) and Dickson et al. (1982) produced and characterised cell cultures grown from dissociated DRG and brain, from 15-20 week old human foetuses, by using standard methods for animal cell culture, and with the aid of cell-type specific markers (Section 1.2.8).

Only very recently have successful cultures of dissociated foetal human spinal cord been reported (Kato et al., 1985; Kim et al., 1986). These have both been produced from very young human foetuses (approx. 8 weeks "in utero"), and have been cultured in serum supplemented medium.

Cultures of human spinal cord neurones are required in order to repeat and extend studies already performed using animal material. In particular, studies on growth factors and disease processes are of interest. Touzeau and Kato (1986) have shown that three key enzymic activities

are unaffected by growth in media supplemented with heat-inactivated (HI) serum from patients with Motor Neurone Disease (MND) (see also Section 1.3.5(iv)).

1.1.3 MEDIA REQUIREMENTS

The earliest culture media were biological fluids, such as lymph, embryonic extract, plasma or serum. Development of synthetic media began with salt solutions which were supplemented with the various biological fluids. Explants and cell dissociates were usually grown embedded in a supporting structure, such as a plasma or fibrinogen clot.

A number of cell lines were isolated which were able to grow directly on the surface of the glass vessel. One example, isolated by Scherer et al. (1953), from a human uterine carcinoma (strain HeLa) was found by Eagle (1955) to proliferate in a medium consisting of a mixture of thirteen amino acids and seven vitamins, together with cofactors, carbohydrate and salts. He was further able to establish the concentration of each constituent required for maximal growth. A small serum supplement of approximately 0.25% was, nevertheless, found to be essential for growth and survival.

Several other media were formulated in the 1950's to allow for the optimum growth of different cell lines and primary cultures. The Dulbecco-Vogt modification of Eagle's medium (DMEM) is richer in glucose than Eagle's minimum essential medium (MEM, BME). For culture of neural tissue, this concentration (4.5g per litre) is usually raised still further (to approximately 6g per litre) by supplementation in individual laboratories (Digby et al, 1985). These media contain bicarbonate which acts as a buffer when exposed to 5-10% CO_2 in

an "open-culture" incubator. Alternatively HEPES buffer may be used in some systems. Sodium phenol red gives a direct visual indication of the pH of the media.

To date, the majority of cultures have been grown in one of the above mentioned media, usually supplemented with approximately 10% animal sera (foetal calf or horse).

A nutritionally complete medium containing, in addition, eight trace elements was formulated by Ham (1965). This medium, F12, was used to great effect by Hutchings and Sato (1978) to define the contribution of serum to the growth of the HeLa cell line. They showed that the serum component of the medium could be replaced by the hormones insulin, transferrin and hydrocortisone plus epidermal growth factor (EGF, see Haigler, 1983, for review) and fibroblast growth factor (FGF, Gospodarowicz, 1975).

These requirements were found to differ from cell line to cell line, and in 1979 Bottenstein and Sato defined a specific serum-free supplemented media for the growth of a neuroblastoma cell line.

The next year they published the first report of the maintenance of post-mitotic primary neurons (chick DRG sensory neurons) in a serum-free modified medium (Bottenstein et al., 1980). Synthetic basal medium was supplemented with insulin, transferrin, progesterone, putresceine and selenium (N1 supplement). They also found that nerve growth factor, NGF (see Bradshaw & Young, 1976, for review) was essential and had to be added to both serum-free and control serum-supplemented medium. An initial period of 48 hours in serum-supplemented media

was allowed for the adequate attachment of the cells to the collagen substrate.

The ability of primary neurones to grow in serum-free chemically defined media has several advantages. In the absence of serum, the non-neuronal cells present fail to multiply and may eventually die off (Bottenstein et al., 1980; Bottenstein, 1983). This means that the cultures are effectively "neurone-enriched" and the neurones can achieve their full developmental potential. The interfering or masking chemical interplay between the neurones and glial cells is reduced, allowing the biochemical parameters and responses of the neurones themselves to be more accurately assessed.

An alternative "in-culture" method of reducing non-neuronal growth in serum-supplemented media is to add mitotic inhibitors (cytosine arabinoside, uridine, fluorodeoxyuridine etc.) to the media at appropriate times (eg. Godfrey et al., 1975). Although these drugs cannot affect the division of the post-mitotic neurones, there is some evidence that they have other undesirable non-specific cytotoxic or biochemical modulatory activities (Kato et al., 1985), and their use is thus best avoided.

Scott and Fisher (1970) demonstrated a 10 fold increase in neurone survival in cultures of dissociated chick DRG when the K^+ ion concentration was raised from 6.8mM to 40.8mM (with reduction in NaCl to maintain the correct osmolarity). Scott (1971) also reported the optimum K^+ concentration for culture of human dissociated spinal ganglia to be 20.8mM.

Bennett and White (1979) showed enhanced survival of

parasympathetic neurones from chick ciliary ganglia (CG) when the K^+ concentration was raised to 25mM without compensatory changes in NaCl. The effect is assumed to result from depolarisation induced by the high K^+ concentration which mimics the normal *in vivo* innervation of the neurones.

These studies have led to the suggestion that dissociated neural tissue should be routinely cultured in high K^+ concentration media. This has been employed by Kato *et al*. (1985), who recorded a twofold increase in choline acetyltransferase (ChAT) activity when human foetal spinal cord cells were grown in 23.6mM potassium chloride (KCl) instead of the usual concentration of 5.4mM.

1.1.4 PURIFICATION OF NEURONES FROM HETEROGENOUS CELL POPULATIONS

Early attempts to purify neurones for culture from mixed cell populations exploited the differential adhesiveness of the cells for material surfaces. Thus, freshly dissociated cells are plated out and left until the non-neuronal cells have attached. Gentle agitation can then be applied to selectively detach the neurones for re-plating (McCarthy & Partlow, 1976; Adler et al., 1979).

Neurones can also be partially purified prior to initial seeding by virtue of their characteristic buoyant densities in low "g" velocity sedimentation (Berg & Fischbach, 1977) or density gradient centrifugation (Schnaar & Schaffner, 1981). The "large cell fractions" (LCF's) thus obtained from spinal cord dissociates show increased ChAT activity and will only survive in culture if they are plated onto a highly adhesive substratum in the presence of muscle cells, or muscle-cell conditioned medium. They develop a primarily motor neuron-like morphology.

It is also possible to purify neurones by virtue of their unique cell surface features. For example, chick embryo sympathetic ganglion neurones possess high numbers of receptors for α-bungarotoxin (α-BgT). Dvorak et al. (1978), used an α-BgT affinity column and succeeded in attaining a purification of 9-10 fold with the final cell suspension being greater than 95% neurones. The cells were viable and electrically active when grown in

culture (with NGF and serum included in the medium).

Immunoglobulins purified from antisera, or monoclonal antibodies specific to neuronal cell surface antigens can also be used as ligands in immunoaffinity columns to effect the purification of neurones (Au & Varon, 1979; Varon & Manthorpe, 1980).

In an alternative approach, antisera or monoclonal antibodies specific for non-neuronal cell surface antigens can be used to destroy unwanted cells selectively through complement-mediated lysis (Varon et al., 1979; Brockes et al., 1979).

In yet other approaches, motor neurones have been purified 4.8 fold from adult mouse spinal cord (Eagleson & Bennett, 1983). The neuronal cell bodies were labelled by the retrograde transport in vivo of True Blue fluorescent dye and horse-radish peroxidase (HRP). Following dissociation of the spinal cord cells, they were sorted by a Coulter counter on the basis of both cell size and True Blue fluorescence. Forty percent (40%) of the "large, fluorescent cell fraction" in subsequent culture were labelled by developing the HRP reaction after 2 hours. However, only 38% of these cells were still viable after a mere 24 hours in culture (the longest period studied). Plating the cells out in skeletal muscle-cell conditioned medium increased the viability of the labelled cells to 70% after 24 hours in culture.

The retrograde labelling technique for motor neurones has also been applied to foetal rats by Smith et al.(1986), who kept 14 day beheaded rat embryos in organ culture for up to 6 hours following the intra-limb injection of

wheat germ agglutinin conjugated to the fluorescent marker Lucifer Yellow Vinylsulfone. Ventral spinal cord cultures were then prepared from the embryos and the labelled neurones were examined *in vitro*. Pre-culture purification based on fluorescence activated cell sorting is, in principle, possible but was not attempted.

To date, there is no specific immunological cell-surface marker for motor neurones. This undoubtedly holds the promise of being the best tool for their eventual purification to homogeneity.

1.1.5 ATTACHMENT OF CELLS AND OUTGROWTH OF NEURITES ON VARIOUS SUBSTRATA

Nerve tissue was originally grown embedded in a plasma or fibrinogen clot, contained in a glass chamber. Nowadays, it is invariably grown in purpose-made plastic ware or on glass coverslips or slides contained therein. A number of additional treatments to such surfaces have been proclaimed beneficial to nerve cell attachment and neurite outgrowth (see Grinnell, 1983, for review).

Popular and efficient factors are those likely to occur naturally in the extracellular matrix, especially collagen (Iversen, 1981) and fibronectin (Steele & Hoffman, 1986).

Synthetic, polycationic substances such as Poly Ornithine (PORN) and Poly-(D/L)-lysine are also commonly used (Collins, 1978; Varon et al., 1979; Bennett & Nurcombe, 1979; Bennett et al., 1980; Smith & Appel, 1983). In addition to improving attachment, these synthetic factors also appear to reduce the proliferation of glia and fibroblasts (Varon, 1977). Because of their property of prompting rapid neural cell adhesion, they are also moderately successful in reducing spontaneous aggregation following initial seeding and thus provide cultures with an improved monolayer-like morphology (Adler et al., 1979). Worthy of a mention in this context is the observation of Thierey et al. (1977), that antibodies to the neural cell adhesion molecule, N-CAM(Brackenbury et al., 1977) inhibit nerve cell aggregation. Unfortunately the presence of anti-N-CAM antibodies in tissue culture

has been observed to interfere with the normal pattern of retinal cell growth and development (Rutishauser et al., 1978, reviewed by Rutishauser, 1984).

Pre-treatment of surfaces with serum-supplemented media has also proved to be beneficial, whether or not the material surface has been coated with one of the other factors (eg. collagen). This effect was originally thought to arise from direct adsorption of fibronectin from the serum component. However, at the serum concentrations normally used (10-20%), it is serum albumin which is adsorbed onto the material surface and this has been shown to inhibit the attachment of certain cell types (Grinnell & Feld, 1982).

It can be seen that there may be a multiple sandwich layer of adhesive factors and cells between the material surface and the neurones themselves. The neurones presumably attach to some factor/s via specific cell surface receptors, and the identity of this factor/s is of paramount importance and interest, with considerable implications both in vitro and in vivo. Liesi et al. (1984) and Calof and Reichardt (1985) have clearly shown that laminin adsorbed onto culture surfaces is the essential component for nerve cell attachment and neurite outgrowth. Laminin is currently receiving much attention in the field of growth cone advancement during embryogenesis and nerve axon regeneration (reviewed by Davis et al., 1985). For instance, it is well known that, in contrast to the PNS, nerve axons will not regenerate in the CNS following section. CNS neurones will, however, regenerate

in the frog and goldfish. This was originally thought to result from an intrinsic property of mammalian CNS neurones, but it now appears that it is the microenvironment which determines whether or not neurite growth will occur (Carbonetto, 1984). Thus, both mammalian CNS and PNS neurones will send axons through living or dead explants of peripheral (sciatic) nerve in tissue culture, but neither will penetrate explants of central (optic) nerve trunk (Schwab & Thoenen, 1985). There is evidence that laminin is only transiently produced by astrocytes during the embryonic establishment of non-regenerating CNS systems eg. chick optic nerve (Cohen et al., 1987), and that astrocytes only continue to produce laminin in mature regenerating systems (Smith et al., 1986). Schwann cells, however, appear to always produce laminin (Longo et al., 1984; Bunge & Bunge, 1983).

There also appear to be non-permissive factors in the CNS. If glial cells are established first in culture and neurones added later (7 days), they will not grow over oligodendrocytes and "windows" are seen (Schwab&Caroni, 1987).It has been determined that myelin is the non-permissive factor and that elastase abolishes this effect, thus indicating a protein component. On sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), two regions of non-permissive proteins have been identified in the region of 30-35KDa and 250KDa.

Neurite outgrowth and regeneration therefore appears to depend on the presence of permissive substrates (esp. laminin) and the absence of non-permissive substrates.

In addition, specific soluble neurotrophic and neuronotropic factors may be involved (Section 1.1.7).

In a heterogeneous cell population, such as that obtained from dissociation of embryonic rat spinal cord, fibroblasts and glial cells are present. Fibroblasts have been shown to secrete fibronectin directly onto material surfaces (Grinnell & Feld, 1982). Glial cells produce laminin. It may therefore be unneccessary to pre-treat material surfaces for this type of culture; a possibility which has been explored in the present thesis.

1.1.6 POLYPEPTIDE GROWTH FACTORS (PGFs)

James and Bradshaw (1984) have published an excellent review on this subject. Briefly, there is now a long list of PGFs which have been recognised, some of which are completely characterised eg. epidermal growth factor (EGF)(reviewed by Haigler et al., 1983), platelet-derived growth factor (PDGF)(Antoniades et al., 1979; reviewed by Westermark et al., 1983), glial growth factor (GGF) (Lemke & Brockes, 1983) and fibroblast-derived growth factor, FDGF(Dicker et al., 1981).

As a general rule PGFs are polypeptides which are produced by one cell type and have certain stimulatory actions, usually on neighbouring cells (paracrine) or even on themselves (autocrine). The effects are usually to stimulate mitogenesis in dividing cell types, to produce an increase in cell size (hypertrophy) or to extend cell survival.

The trophic effect of nerve on muscle has long been recognised. As a result of denervation of adult muscle there is a decrease in the resting membrane potential, development of extrajunctional acetylcholine sensitivity, loss of endplate cholinesterase, and muscle atrophy and degeneration. The original concept that muscle activity alone could regulate these processes, is no longer accepted (reviewed by Oppenheim, 1985). Podleski et al. (1978) added rat spinal cord explants or CNS homogenates to a cloned rat muscle cell line and found a large increase in the density and numbers of acetylcholine receptors. Markelonis and Oh (1979) extended these studies

to show that a protein of molecular weight 84KDa extracted from chicken sciatic nerve had trophic effects on primary cultures of chick muscle cells. The rate and degree of morphological maturation and the level of protein synthesis in the cells was enhanced. Muscle fibres were also reported to survive for longer in the presence of this extracted protein. They further went on to identify and name the glycoprotein responsible "sciatin" and to show that it also had effects on the number and density of acetylcholine receptors (Markelonis et al., 1982a). It appears that "sciatin" is closely related to transferrin, another growth-regulating protein (Markelonis et al., 1982b).

Fontaine et al. (1986) also have identified a peptide in spinal cord motoneurones, encoded by the calcitonin gene and hence named calcitonin gene-related peptide (CGRP), which increases the number of acetylcholine receptors in primary cultures of chick embryo myotubes.

It is likely that at least one of the factors identified is a true polypeptide growth factor in that it has long-term effects on the growth and survival of muscle cells. Other factors, which have more limited and shorter term effects, especially on receptor function etc. will probably best be classified along with other similarly acting peptides termed collectively the "Neuropeptides" (Bloom, 1979).

1.1.7 SPECIFIC NEURONOTROPHIC FACTORS

As mentioned previously (Section 1.1.5) there is a group of factors which promote neurone attachment to the substratum and/or have neurite-outgrowth promoting activity (NOPA). There is also a group of soluble factors which have marked effects on neuronal growth, development, differentiation and survival (reviews, Varon & Bunge, 1978; Varon & Adler, 1981; Barde et al., 1983).

The earliest of these to be recognised was a protein, originally isolated from snake venom, which specifically promoted the growth of sensory and sympathetic nerve cells both in tissue culture and in the living chick embryo (Cohen, 1959).

The mouse sub-maxcillary gland was found to be a rich source of this nerve-growth promoting protein (Cohen, 1960) which was subsequently purified 100 fold with a 10-20% yield. An antiserum was prepared to this fraction and daily subcutaneous injections were given to newborn or adult mice. This resulted in a rapid and extensive destruction of the sympathetic chain ganglia (Levi-Montalcini & Booker, 1960). In newborn mammals, daily injection of the antiserum for a period of 8 days resulted in the disappearance of up to 99% of the sympathetic nerve cells. This phenomenom was termed "immunosympathectomy".

The protein itself was named nerve growth factor (NGF) although this term is misleading as some groups of neurones are unaffected by the factor or its antiserum (notably the somatic motor neurones).

Since then, many aspects of NGF have been extensively studied and periodically reviewed (Levi-Montalcini & Angeletti, 1968; Mobley et al., 1977; Greene & Shooter, 1980; Thoenen & Barde, 1980; Harper & Thoenen, 1980). Briefly, the "active" component of NGF (beta or 2.5S NGF) is a dimer of two identical polypeptide chains. Each chain is composed of 118 amino acids, with a molecular weight of 13,259 Da. There are three intra-chain disulphide bridges. Sequence analysis has shown close analogy with insulin and even more so with proinsulin, thus indicating a common evolutionary precursor. In the submaxcillary gland βNGF occurs in a complex (7S NGF) with two other subunits (gamma and alpha). It is widely distributed and is found particularly in target tissues for sympathetic and sensory neurones. Traces also occur in blood (reviewed by Purves, 1986). In vitro, NGF has been shown to have many effects on the metabolism of these neurones. It stimulates anabolic processes such as uridine uptake, synthesis of RNA and protein, lipid, and glucose uptake and metabolism. Administration of NGF to cultured superior cervical ganglia (SCG) of neonatal rat produced a five fold increase in tyrosine hydroxylase and dopamine β-hydroxylase levels; activity which resulted in increased noradrenaline synthesis (see Thoenen & Barde, 1980).

NGF administration also increases neurite outgrowth and neuronal survival, although Burnham et al. (1972) did show that NGF could be replaced to some extent by an increase in the proportion of non-neuronal ganglionic cells. Lazarus et al. (1976) also showed that sympathetic

neurones could subsequently survive without NGF supplements, if they received it for the first 5-7 weeks in culture.

More specifically, McGuire et al. (1978) demonstrated that NGF was capable of stimulating the incorporation of radiolabelled fucose or glucosamine into a cell surface glycoprotein of the rat pheochromocytoma PC12 cell line. This glycoprotein was accordingly dubbed NGF-inducible large external (NILE) glycoprotein, and was shown to be immunologically distinct from the large external transformation-sensitive (LETS) glycoprotein of Hynes (1976), although it may be identical to part of the L1 antigen (Sajovic et al., 1986). Remarkably the morphological effect of the NGF on the cell line is that the cells stop dividing, extend long microtubule-containing processes and acquire electrical excitability and increased sensitivity to acetylcholine. They appear to have been "converted" to sympathetic neuron-like cells.

The site and mechanism of action of NGF is not totally clear, although it has been demonstrated that it is endocytosed by a receptor mediated process and transported retrogradely to the neuronal soma (Stoeckel & Thoenen, 1975). Some of its effects may be more locally controlled via second messengers (Varon & Bunge, 1978).

In contrast to the PNS, catecholaminergic neurones in the CNS do not respond to NGF (Gnahn et al., 1983; Hefti et al., 1985). In particular, ChAT activity in the corpus striatum of neonatal rats is increased two fold by direct intracerebroventricular injection of

NGF (30μg every 2 days) (Mobley et al., 1985).

Furthermore, Fischer et al. (1987) have experimented with continuous intracerebral infusion of NGF (over four weeks) into aged rats with behavioural impairment resulting from cholinergic neuron atrophy. They report that this treatment resulted in the "amelioration of cholinergic neuron atrophy and spatial memory impairment". The implications from this and other data, as also mentioned by Korsching (1986), is that it is possible that Alzheimer's disease may be the result of an impairment in a retrograde trophic support system for the cholinergic neurones involved.

Some of the above data are consistent with the concept of Varon & Adler (1981) that NGF may "control the quantity of neurotransmitter produced regardless of its choice". It indicates the involvement of other "specifying" factors.

By analogy with NGF it has long been suspected that there are other trophic molecules responsible for the maintenance of neuronal populations unaffected by NGF itself (eg. spinal motor neurones). Research in this field has been slow, because of the lack of a recognised rich source of the factor/s.

Hamburger (1975) reported that during normal development of the chick embryo there is a loss of approximately 40% of the neurones from the lateral motor column (LMC). This loss occurs between days 6.5 and 9.5 which is also the time that the motor nerve axons are forming connections with target skeletal muscle. Hollyday

and Hamburger (1976) further showed that the loss of neurones could be increased by removal of a limb bud or buds from the developing chick embryo, or decreased by grafting on an additional leg. This was an early indication that motor neurones must make connections with their peripheral targets in order to survive.

One suggestion as to the mechanism involved was that the nerve fibers competed for a limited supply of a trophic agent supplied by the muscle cells. Once a connection was made and the trophic agent sequestered, then subsequent attempts at innervation by other nerve fibers would be unsuccessful. Support for such a situation, by analogy, comes from sympathetic neurones, death of which, caused by axotomy (Banks & Walters, 1977) or vinblastine administration (Johnson, 1978) can be prevented by the exogenous administration of NGF. NGF can also reduce naturally-occurring nerve cell death in the chick spinal cord autonomic visceral motor columns by more than 60% (Oppenheim et al., 1982).

Giller et al. (1973) reported "the first demonstration that postsynaptic cells may specifically increase the pre-synaptic cell enzyme activity responsible for the neurotransmitter used at that junction". Using foetal mouse spinal cord and skeletal muscle, they showed an approximate 10 fold increase in ChAT activity when the cells were cultured together rather than separately. Their data "suggested that the increased ChAT activity was due to a trophic effect of muscle on spinal cord neurones".

They went on to show that the active factor could be transmitted in the medium from cultured muscle cells when applied to cultured nerve cells (Giller et al., 1977).

Parallel studies (White & Bennett, 1978; Bennett & Nurcombe, 1979) with cholinergic ciliary ganglion (CG) neurones demonstrated increased initial survival of the neurones when cultured with skeletal myotubes or in muscle cell conditioned media.

Nurse and O'Lague (1975) demonstrated that sympathetic neurones from the newborn rat superior cervical ganglion (SCG) were capable of developing functional cholinergic synapses with skeletal myotubes in culture. This was surprising as the neurones in vivo are destined to be noradrenergic.

Patterson and Chun (1977) suggested that autonomic neurones are capable of being either adrenergic or cholinergic. They showed a 25 fold depression in catecholamine production linked with a 40 fold increase in acetylcholine synthesis when SCG neurones were cultured in skeletal muscle cell conditioned media. Neuronal survival and overall growth was apparently unaffected.

Ross et al. (1977) examined this finding with SCG explants of various ages and came to the conclusion that the ability of the adrenergic neurones to develop cholinergic characteristics "reaches a maximum at approximately the second postnatal day: beyond the third postnatal week this shift does not occur".

In the last ten years there has been a veritable

explosion in the testing of conditioned media and tissue extracts on neuronal culture systems. The conditioned media are prepared from a variety of different cell lines and primary cultures including glial, striated muscle, smooth muscle, liver, lung etc. from several different species. Likewise, extracts of various tissues from foetal, postnatal and normal or denervated adult animals are prepared. They are tested for potential neuronotrophic activity on various neuronal cultures eg. autonomic, sensory or somatic motor neurones.

The activities studied are:

(a) ability to improve attachment of neurones to the substratum (assessed after a few hours in culture by counting adherent cells),

(b) ability to promote initial neuronal survival (assessed after several hours or up to a few days in culture by counting viable cells),

(c) ability to promote neurite outgrowth from cells or explants in culture (assessed after the first few hours/days in culture by counting the number of neurites and/or measuring their length, or by assaying neurofilament protein levels),

(d) ability to stimulate specific neurotransmitter synthesis (usually assessed by assaying neurotransmitter production in terms of the levels of the synthesising enzyme eg. Acetylcholine production by ChAT activity).

The ability of conditioned media or tissue extracts to promote long term maintenance of neurones in culture has not been generally assessed, because of the

unsuitability of most culture systems for this purpose (reviewed by Appel, 1984). Thus, most culture systems have serum included in the medium which undoubtedly contains directly or indirectly acting (eg. via glial cell mediation) maintenance factors. The inclusion of serum in the medium may also affect the other parameters studied, which could account for the considerable variation in levels of stimulation reported.

Table 2 (p 28) is a compilation of reports associated with neuronotrophic activity of conditioned media and tissue extracts (reviews are not included).

It appears that there may be three types of "cholinergic activity". Firstly, an "instructive" activity which is capable of specifying the differentiated fate of some autonomic neurones (reviewed by Fukada, 1985) and may or may not be subserved by the same molecule which promotes the second activity, namely the increased levels of choline acetyltransferase (ChAT) and hence acetylcholine synthesis. The third activity involves the selective survival of cholinergic neurones in a heterogeneous cell population, which may be subserved by yet another molecular species.

The cholinergic activities appear to be connected with any tissue which itself receives a cholinergic innervation in vivo. The ChAT "level stimulating" activity may also be found in some other tissues eg. liver, serum. The activities are usually associated with glycoproteins of varying molecular weights eg. 14-17KDa (Smith et al., 1985)), 21KDa (Weber et al., 1985), 22KDa (Fukada,

Table 2 Cumulated reports of neuronotrophic activities associated with co-culture, conditioned media, and tissue extracts (Chronological order 1973-1987 inclusive).

Reference	Extract or Cond. media or co-culture	Tissue or cell line	Source species	Neuronal test system	Source species	Substrate	Basal medium	Imp. suppls	Time studied	Activity studied	Result
Giller et al. 1973	co-culture	skeletal muscle	mouse embryo	diss. spinal cord	mouse embryo	coll-agen	D-VMEM	10%HS U/FdU	up to 20 day	ChAT	10x inc.
Ross & Bunge,1976	co-culture	atrium	rat	explant S.C.G.	rat	?	?	?	30 days	ChAT	2x inc.
Patterson & Chun, 1977	co-culture or cond. media	various tissues or cell line	rat neonate	diss. S.C.G.	rat neonate	coll-agen	L-15	10%FCS	22 days	ChAT	100-1,000 x inc.

(continued)

Table 2 (continued)

Reference	Extract or Cond. media or co-culture	Tissue or cell line	Source species	Neuronal test system	Source species	Substrate	Basal medium	Imp. suppls	Time studied	Activity studied	Result
Giller et al, 1977	co-culture or cond. media	heart or skeletal muscle	mouse neonate	diss. spinal cord	mouse embryo	collagen	D-VMEM or MEM	10%HS 10%FCS U/FdU	up to 21 days	ChAT	up to 20x inc.
White & Bennett, 1978	co-culture	skeletal muscle	chick embryo	diss. C.G.	chick embryo	collagen	E.B.M. & 10% HBSS	10%FCS	up to 6 days	initial survival	inc.100%
										neurite outgrowth	greatly inc.
Collins 1978	cond. media	heart	chick embryo	diss. C.G.	chick embryo	PORN ± cond. media	HAMS FI2	10%FCS	up to 12 h	neurite outgrowth	greatly inc.

(continued)

Table 2 (continued)

Reference	Extract or Cond. media or co-culture	Tissue or cell line	Source species	Neuronal test system	Source species	Subs-trate	Basal medium	Imp. suppls	Time studied	Activity studied	Result
Popiela et al. 1978	cond. media	skeletal muscle	chick embryo	diss. spinal cord	chick embryo	-	-	10%FCS or 10%DHS	11 days	ChAT	up to 5x inc.
Varon et al. 1979	cond. media	heart	chick embryo	diss. C.G.	chick embryo	PORN or coll-agen	EBM	10%FCS or 10%DHS	24h	initial survival	inc.
	extract	whole embryo	chick							neurite outgrowth	inc.
										ChAT	inc.
Nishi & Berg, 1979	cond. media	heart or skeletal muscle	chick embryo	diss. C.G.	chick embryo	coll-agen	MEM	10%DHS 5%CEE	up to 15 days	survival in culture	inc. from 2-15 days
Bennett & Nurcombe 1979	cond. media	heart or skeletal muscle	chick embryo	diss. C.G. and explant	chick embryo	PORN	EBM	10%FCS	up to 4 days	initial survival	50% inc.
										neurite outgrowth	dramatic inc.

(continued)

Table 2 (continued)

Reference	Extract or Cond. media or co-culture	Tissue or cell line	Source species	Neuronal test system	Source species	Subs-trate	Basal medium	Imp. suppls	Time studied	Activity studied	Result
Ebendal et al 1979	extract & purified 40KDa	heart	chick embryo	co-cultured spinal, sympath-etic & ciliary ganglia	chick embryo	coll-agen	EBM	1%FCS	2 days	neurite outgrowth	greatly inc.
Adler & Varon 1980	cond. media	heart	chick embryo	diss. C.G.	chick embryo	coll-agen	BME	10%FCS	24h	initial survival	inc. on both substrata
										neurite outgrowth	inc. on PORN only
Bennett et al 1980	cond. media and co-culture	skeletal muscle	chick embryo	diss. spinal cord	chick embryo	poly-L-lysine	EBM	10%FCS	2½ days	initial survival HRP-labelled m.neurons	inc. approx. 80%

(continued)

Table 2 (continued)

Reference	Extract or cond. media or co-culture	Tissue or cell line	Source species	Neuronal test system	Source species	Subs-trate	Basal medium	Imp. suppls	Time studied	Activity studied	Result
Brookes et al. 1980	cond. media	skeletal muscle	mouse neonate	diss. spinal cord	mouse embryo	coll-agen	MEM	10%FCS 10%DHS U/FdU	up to 21 days	ChAT	up to 7.7x inc.
Godfrey et al. 1980	cond. media	various tissues & cell lines	mouse rat chick embryo	diss. spinal cord	mouse embryo	coll-agen	DMEM	10%FBS 10%HS U/FdU	up to 14 days	ChAT	up to 3.7% inc with mouseMCM
Schnaar & Schaffner 1981	cond. media	skeletal muscle and G-8 cell line	chick embryo	purified moto-neurons from diss spinal cord	chick rat embryo	coll-agen or poly-L-lysine	DMEM	10%FBS 10%DHS 2%CEE	up to 12 days	initial survival	MCM & poly-L-lysine essential
										ChAT	inc. 3-4x

(continued)

Table 2 (continued)

Reference	Extract or cond. media or co-culture	Tissue or cell line	Source species	Neuronal test system	Source species	Substrate	Basal medium	Imp. suppls	Time studied	Activity studied	Result
Nishi & Berg 1981	Extract and purified 20KDa 50KDa	Eye	chick embryo	diss. C.G.	chick embryo	fibroblast material	MEM	10%DHS +20mM K^+	20 days	neuronal growth	inc. by 20KDa factor
										ChAT activity	inc. by 50KDa factor
Henderson et al 1981	purified cond. media 40 & 500 KDa	skeletal muscle	chick embryo	diss. spinal cord	chick embryo	untreated plastic	MEM	serum free	20h	neurite outgrowth	inc. 3x
Barde et al. 1982	purified BDNF 12,300Da	brain	pig adult	diss. D.R.G.	chick embryo	-	F14	10%DHS	48h	initial survival	greatly inc.

(continued)

Table 2 (continued)

Reference	Extract or cond. media or co-culture	Tissue or cell line	Source species	Neuronal test system	Source species	Substrate	Basal medium	Imp. suppls	Time studied	Activity studied	Result
Slack & Pockett 1982	extract	skeletal muscle (denerv-ated)	mouse adult	diss. spinal hemi-cord HRP-labelled moto-neurons	chick embryo	coll-agen	BME	11.1% FCS	2 days	initial survival	inc. 3.6x
Dribin & Barrett 1982	cond. media & purified 50KDa glyco-protein	muscle or fibro-blast cultures	rat foetus	explant spinal cord	rat foetus	coll-agen & poly L-lysine	modif. L-15	-	6 days	neurite outgrowth	mean inc.by 2x
Tanaka et al. 1982	serum extract cond. media HMW/LMW	brain skeletal muscle heart liver	chick neonate	explant spinal cord	chick embryo	coll-agen or PORN	MEM	serum or extract or nil	up to 7 days	neurite outgrowth	greatly inc. by FCS & cond. med. esp. on PORN

(continued)

Table 2 (continued)

Reference	Extract or cond. media or co-culture	Tissue or cell line	Source species	Neuronal test system	Source species	Subs-trate	Basal medium	Imp. suppls	Time studied	Activity studied	Result
Kligman 1982	Extract and purified 75KDa (37KDa reduced)	brain	bovine	diss. cerebral cortex neurons	chick embryo	poly-L-lysine	HAMS F12	modif. "N1" suppl. + 0.02% FCS	4 days	neurite outgrowth	induced at 100ng/ml (purified fraction
Collins & Dawson 1982	cond. media	heart cell culture	chick embryo	diss. C.G.	chick embryo	PORN & subs-tratum NOPF	HAMS F12	10%FCS	up to 36h	neurite elong-ation	inc. x3
Tanaka & Obata 1984	extract & cond. media 150,70 & 40KDa	various tissues	chick neonate	spinal cord (diss)	chick embryo	PORN	MEM	serum free	up to 7 days	initial survival	inc. by extract & cond. media
										neurite outgrowth	only inc. by cond. media

(continued)

Table 2 (continued)

Reference	Extract or cond. media or co-culture	Tissue or cell line	Source species	Neuronal test system	Source species	Subs-trate	Basal medium	Imp. suppls	Time studied	Activity studied	Result
Manthorpe et al 1983	cond. media	RN22 schwan-noma	rat	diss. spinal cord low or high density	chick embryo	PORN	EBM	0.5% ovalb-umen	1 or 5 days (toxic effect)	initial survival	low density 50% inc. 1 day
										ChAT	slight inc.
Eagleson & Bennett 1983	cond. media	skeletal muscle	chick embryo	diss. spinal cord HRP-labelled moto-neurons	mouse adult	poly-L-lysine	EBM & HANKS SALTS	10%DHS	24h	initial survival	32% inc.
Smith & Appel 1983	extract	skeletal muscle cardiac brain	rat neonate (3wks)	diss. ventral spinal cord	rat foetus	poly-L-lysine	DMEM	10%DHS cyto-sine arabin-oside	up to 4 days	neurite outgrowth	inc. by extract SK-M only
										ChAT	inc 2-3x all extracts

(continued)

Table 2 (continued)

Reference	Extract or cond. media or co-culture	Tissue or cell line	Source species	Neuronal test system	Source species	Substrate	Basal medium	Imp. suppls	Time studied	Activity studied	Result
Nurcombe et al. 1984	extract	skeletal muscle denerv-ated comp.to inner-vated	rat adult	explant spinal cord or diss HRP-labelled motor-neurons	chick embryo	poly-L-lysine	MEM	5%DHS 48-72h then SFM ± extract	up to 96h	initial survival	inc. up 60% diss S.C.
										neurite outgrowth	greatly inc. explants
Barbin et al. 1984	extract & purified CNTF (20.4KDa)	eye (CIPE)	chick embryo	diss. C.G.	chick embryo	PORN & PNPF	HEBM	10%FCS	24h	short term survival	CNTF supports 60-90% survival
Doherty et al. 1984(c)	cond. media	skeletal muscle biopsy	human infant	spinal cord & DRG	chick embryo human foetus	coll-agen	DMEM	serum free	up to 5 days	neuro-filament express-ion mAb binding	inc. in both spinal cords not DRG
Calof & Reichardt 1984	cond. media. two activ-ities	skeletal muscle & spinal cord & fibro-blasts	chick embryo	purified spinal moto-neurons	chick embryo	poly-L-lysine	DME	modif. "N1" suppl. or 3%DHS	up to 6 days	neurite outgrowth	greatly inc.
										cell survival	also enhanced

(continued)

Table 2 (continued)

Reference	Extract or cond. media or co-culture	Tissue or cell line	Source species	Neuronal test system	Source species	Subs-trate	Basal medium	Imp. suppls	Time studied	Activity studied	Result
Flanigan et al. 1985	extract	skeletal muscle skin liver	chick embryo	diss. spinal cord semi-purified motor-neurons	chick embryo	coll-agen	DMEM	10%HS 10%FCS	up to 5 days	initial survival	1.6xinc. in skeletal muscle ext. only
										ChAT	up to 2x inc. as above
Weber et al. 1985	cond. media purified 21 KDa	skeletal muscle	rat neonate	diss. SCG	rat neonate	-	L15	5% rat serum vitamin mix. NGF cytosine arabin-oside	up to 15 days	ChAT	up to 9x inc. purified
Kaufman et al. 1985	cond. media & extracts purified (40KDa)	skeletal muscle	rat adult neonate	diss. spinal cord	rat foetus	coll-agen & poly-L-lysine	CSF-like nutr-ient medium	purified survival factor from DHS	up to 12 days	Ach synthesis	enhanced
										ChAT	"
										protein synthesis	"
										GABA synthesis	dec.

(continued)

Table 2 (continued)

Reference	Extract or cond. media or co-culture	Tissue or cell line	Source species	Neuronal test system	Source species	Substrate	Basal medium	Imp. suppls	Time studied	Activity studied	Result
Steele & Hoffman 1985	extract & purified 160,195 & 200KDa	skeletal muscle	calf foetus	diss. DRG	chick embryo	poly-L-lysine laminin fibronectin	DMEM or HAMS F12 & hormones	10%FCS NGF	up to 72h	neurite outgrowth	greatly inc.
Calof & Reichardt 1985	cond. media purified laminin	skeletal muscle	chick embryo	purified spinal motoneurons	chick embryo	poly-D-lysine	DME	-	24h	neurite outgrowth	laminin enhanced
Smith et al. 1985	extract purified 35,15 & 60KDa	skeletal muscle	rat foetus neonate	diss. ventral spinal neurons	rat foetus	poly-L-lysine	DMEM	10%DHS & cytosine arabinoside	up to 5 days	morphologic activity	associated with 35KDa glycoprotein
										cholinergic activity	assos. with 15& 60KDa glycoprotein

(continued)

Table 2 (continued)

Reference	Extract or cond. media or co-culture	Tissue or cell line	Source species	Neuronal test system	Source species	Substrate	Basal medium	Imp. suppls	Time studied	Activity studied	Result
Oorschot & Jones 1986	serum	-	calf foetus	explant sensori-motor cortices	rat foetus	poly-L-lysine	DMEM	14 days 20%FCS then 4 days SFM	18 days	neurite outgrowth viability	only maintained in serum
										survival	unaffected
Doherty et al. 1986	cond. media	skeletal muscle biopsy	human infant	diss. DRG or spinal cord	chick embryo	collagen &/or poly-L lysine	DMEM	7.5%HS cytosine arabinoside	up to 5 days	initial survival	2x inc. spinal cord only
										neuro-filament protein	75% inc. spinal cord only
										ChAT	50% inc. as above
Smith et al. 1986	extract purified 1.2-1.5 35 & 55 KDa	skeletal muscle	rat foetus neonate	purified motor neurons	rat foetus	poly-L-lysine	DMEM	10%DHS & cytosine arabinoside	up to 14 days	morphologic activity	35&55KDa motor-neurone selective
										cholinergic activity	55 & 1.2-1.5 KDa
										survival	1.2-1.5 KDa

(continued)

Table 2 (continued)

Reference	Extract or cond. media or co-culture	Tissue or cell line	Source species	Neuronal test system	Source species	Substrate	Basal medium	Imp. suppls	Time studied	Activity studied	Result
Gurney et al. 1986(a)	purified neuro-leukin 56KDa	sali-vary gland	mouse adult	diss. spinal cord & DRG, SCG & CG	chick embryo	PORN & laminin	DMEM	10% low mitogen bovine serum	24h	neurite outgrowth initial survival	no effect doubled in spinal cord & DRG only
Bostwick et al. 1987	purified extract C-CTF	hippo campus	rat pups	explant septal nuclei	rat foetus	?	DMEM	N_2 2%RBS	up to 8 days	ACh synthesis	enhanced
										ChAT activity	moderate inc.
Waters & Hendry 1987	extract purified 22/23KDa	heart	bovine	diss. CRG	chick embryo	collagen	DMEM	10% HS HEPES	24h	initial survival	up to 60% inc.

1985), 40-45KDa (Nishi & Berg, 1981), 55-65KDa (Smith et al., 1985).

The neurite outgrowth promoting activities (NOPA) or "morphologic" activities, are apparently associated with two species. One is of high molecular weight and attaches to the substratum (probably laminin) and is only found in conditioned media (rev. Barde et al., 1983). The other is of lower molecular weight eg. 35KDa (Smith et al., 1985), 40KDa (Henderson et al., 1981), and remains in solution to directly stimulate the cell bodies to put forth growth cones. Glial cells also produce a soluble neurite outgrowth promoting factor of 43KDa which is believed to operate via its protease inhibitory activity (Monard, 1985).

Survival of neurones may also be promoted by other molecules, or any of the above. There may be multiple sources available for different stages in development ie. serum ⟶ glial ⟶ target tissue in that order.

The apparent multiple factors are in marked contrast to NGF which serves many functions in the sensory and sympathetic nervous system (reviewed by Yanker & Shooter, 1982).

Smith et al. (1986) have, however, demonstrated more restricted and selective effects of their muscle-derived neuronotrophic factors. Using cultures of foetal rat ventral spinal cord in which the motorneurones had been specifically labelled by retrograde transport of wheat germ agglutinin conjugated to the fluorescent marker Lucifer Yellow vinylsulfone, they were able to

show increased survival, neurite outgrowth and acetylcholine synthesis in the labelled neurones when grown in medium containing muscle extract. Their data of selective effects of partially purified factors is based on the identification of cholinergic neurones by a monoclonal antibody to choline acetyltransferase. They claim that this identifies motorneurones because "cholinergic cells other than motorneurones in the ventral cord.....are rare at the stage of rat development from which the cultures were prepared". On this basis they have shown that their 55KDa neutral glycoprotein specifically stimulates acetylcholine synthesis and neurite outgrowth of motorneurones, but has no effect on their initial survival, which appears to be increased by a 1200-1500Da peptide (which also has a stimulatory effect on acetylcholine synthesis). The 33-35KDa "morphologic factor" is <u>not</u> specific for motorneurones, but stimulates neurite outgrowth in other neurones as well, while having no effect on acetylcholine synthesis.

The question of long term maintenance of motor-neurones is not addressed in the above study. Data in this respect would help to complete the picture on motor neurone-specific trophic factors and shed light on the question as to whether they rely for their continued survival, in the adult animal, on a regular supply of a single factor which also maintains cholinergic activity. Such a factor might be secreted in larger quantities following denervation so as to stimulate terminal or collateral sprouting and hence re-innervation (Pestronk & Drachman, 1984).

A fascinating recent development has occurred in connection with the 56KDa neuronotrophic factor of Gurney et al (1984a; 1984b).

This factor is also mentioned in the context of Motor Neurone Disease (MND) (Section 1.3.5V).

It was reported to be released by denervated or immobilised skeletal muscle and to induce neuronal sprouting in vivo. The finding of antibodies directed against this protein in the serum of patients with MND was, however, insubstantiated (Hauser et al., 1986; Ingvar-Maeder et al., 1986; Gurney et al., 1986a).

Subsequently, Gurney et al. (1986a; 1986b) identified the same factor in mouse salivary gland. Upon purification and testing on cultured chick neurones it did not show any neurite-outgrowth promoting activity (as might have been expected from the previous data), but instead was active in supporting the survival of a sub-population of spinal and sensory (but not autonomic) neurones. They also identified the factor as a "lymphokine product of lectin-stimulated T cells which induces immunoglobulin secretion by cultured human peripheral blood mononuclear cells". On this basis the factor was named Neuroleukin. Neuroleukin is reported to be present in skeletal muscle> brain >> heart, kidney, testes > liver and salivary gland (in that order of relative content).

It has been proven, through amino acid sequence analysis comparison, that the factor termed "Neuroleukin" by Gurney et al.(1986a) is in fact the enzyme glucophosphoisomerase (GPI)(Faik et al., 1988; Chaput

et al., 1988; Gurney, 1988). This extraordinary finding has yet to be fully explained, although it is possible that the enzyme does act as a neuronotrophic factor through combination with a neuronal surface receptor. A 19-amino acid synthetic peptide has been produced which is part of the gp120 envelope glycoprotein of the human immunodeficiency virus (HIV-1) and also Neuroleukin. It has been shown to block the action of Neuroleukin (Ho et al., 1987) and might therefore help to explain the neuropathology sometimes associated with the acquired immunodeficiency syndrome (AIDS).

1.2 IDENTIFICATION AND ASSAY OF THE DIFFERENT CELL TYPES PRESENT IN NEURAL TISSUE CULTURE

1.2.1 MAJOR CELL TYPES PRESENT IN THE VERTEBRATE NERVOUS SYSTEM

The vertebrate nervous system is a complex organisation of many different cell types which perform highly specialised functions. Whenever neural tissue is used as the source for culture, these cell types will be present to different extents and in varying degrees of organisation according to the system chosen (Section 1.1.1).

In dissociated cell culture it has been demonstrated that the various cell types present re-organise themselves into functional arrangements which are similar, if not identical, to those present in the intact tissue (Nelson, 1975 for review).

The three main types of cells likely to be present in neural tissue culture are neurones, glial cells and connective tissue cells.

Neurones can be divided into many classes and sub-classes eg. somatic motor, visceral motor, sensory, inter-neurones. An important point is whether a neurone can be classified as "central" or "peripheral". A true central neurone is one whose cell body, axon and terminal occur within the CNS eg. an upper motor neurone (UMN). A neurone whose cell body is located within the CNS is also classified as "central", although its axon may leave the CNS and terminate in the periphery eg. a lower

motor neurone (LMN). In embyogenesis both of the above neurones are derived from the neural tube. Neurones of the peripheral nervous system (PNS), however, are generated from the neural crest and possibly also the placodes (Cohen & Selvendran, 1981). They include the autonomic neurones of the superior cervical ganglia (SCG) and the sensory neurones of the dorsal root ganglia (DRG).

Glial cells can also be divided into several classes and sub-classes.

Oligodendrocytes are the cells primarily responsible for myelination in the CNS, Schwann cells are their equivalent in the PNS (Raff et al., 1978).

Astrocytes provide physical and chemical support for the neurones of the CNS. The protoplasmic (TYPE I) astrocytes occur mainly in white matter, whereas the fibrous (TYPE II) astrocytes occur mainly in grey matter where they are closely associated with neurone cell bodies and axons, sending end feet to impinge upon them. They also send end feet to blood vessels and act as intermediates between blood and neurones, thus contributing to the "blood-brain barrier" (reviewed by Abbott, 1986; Janzer & Raff, 1987). In particular, there is good evidence that astrocytes are responsible for controlling the neuronal extracellular K^+ and Cl^- concentration through specific uptake processes, thus playing a role in the ion-flux events which accompany nerve-impulse transmission (reviewed by Abbott, 1986).

Recent evidence (Raff et al., 1983; Miller & Raff,

1984) suggests that type II astrocytes are developmentally more closely related to oligodendrocytes than they are to type I astrocytes - both type II astrocytes and oligodendrocytes have a common precursor cell (the 0-2A progenitor).

The microglial cells are phagocytic scavengers which are now thought to originate from outside the nervous system. They are derived from circulating blood monocytes during embryogenesis and migrate into the CNS as an "amoeboid" form which eventually become static and extend processes. They can resume their phagocytic activity during any period of neural injury. However, if the damage is severe, then there may also be re-invasion of the CNS by the monocyte macrophages (reviewed by Ling, 1981; Perry & Gordon, 1988).

The connective tissue cells are termed fibroblasts and are of mesenchymal origin, deriving from the meningeal capsule of the nervous tissue (Schachner , 1982a,b,c).

1.2.2 MORPHOLOGY OF CELLS IN NEURAL TISSUE CULTURE

It is sometimes possible to distinguish the various cell types purely by morphology. Thus, neurones are likely to be seen as large cell bodies with extensive neuritic trees and long axons. Astrocytes (especially Type II) are named according to their characteristic star-shaped appearance.

It is, however, impossible to distinguish between protoplasmic (Type I) astrocytes and fibroblasts based on purely morphological criteria. Both appear as large, flat cells with a large oval nucleus.

Oligodendroglia, which have extensive branched processes, may be confused for fibrous (Type II) astrocytes. Furthermore, some astrocytes tend to look remarkably like neurones, as some sub-classes have a long axon-like process (Bergmann glia).

It is also becoming clear that some cell types may appear identical, and yet represent different sub-classes which are categorised by surface antigenic differences (Section 1.2.8(ii)).

Amoeboid microglial cells are relatively easy to distinguish as they have no processes, an off-set nucleus, and highly vacuolated cytoplasm.

In view of the difficulties attending unambiguous identification of the various cell types, the search for specific markers has been long and continues unabated. The various methods used are reviewed in the forthcoming sections.

1.2.3 HISTOCHEMICAL TECHNIQUES FOR DISTINGUISHING THE MAJOR CELL TYPES IN NEURAL TISSUE

The main existing methods were reviewed by Humason in 1967. They include a modification of the original Bielschowsky method for silver impregnation of nerve fibres and neurofibrils which appear to be brownish-black. There are many other variations on the silver impregnation theme, including the Golgi Rapid Method which specifically stains nerve cells and processes deep black, while the background remains light yellow. A further method is that of Sevier and Munger (1965) which is suitable for paraffin-embedded sections. The specificity of such methods has, however, always been poor.

Perhaps the most successful and recent silver staining method was reported by Howard and Barnes (1979), who developed a modification of the Del Rio-Hortega silver carbonate method for the demonstration of central nervous system elements, and claimed that "neurones, oligodendroglia, astrocytes and microglia can be differentiated on the basis of their nuclear staining properties". The gold chloride sublimate method of Ramón y Cajal is reputed to be specific for astrocytes which are stained black, in contrast to nerve cells which are pale red (Humason, 1967). The pyridine-silver method of the same author, stains nerve cells and neurofibrils and can distinguish between myelinated and non-myelinated fibres.

Other methods, such as Luxol Fast Blue S and that of Ora (Humason, 1967) rely on the staining of myelin.

Howard and Barnes (1979) stated that methods such as Luxol Fast Blue S, periodic acid Schiff and aldehyde fuchsin had only met with "some success" in their hands.

The doorway has always been open for better, more specific techniques than these early ones.

1.2.4 HISTOCHEMICAL TECHNIQUES FOR THE LOCALISATION OF SPECIFIC NEUROTRANSMITTERS AND ASSOCIATED ENZYMES

An alternative histochemical approach is to stain substances which are likely to be specific to particular groups of cells. In particular, Falck and Owman (1965) developed a fluorescent histochemical technique for the cellular localisation of biogenic amines. The technique relies upon the fact that certain monoamines can be converted to fluorescent products by treatment with paraformaldehyde gas, provided that they are enclosed in a dry protein layer. Noradrenaline,5-hydroxytryptamine and dopamine are all amenable to this treatment, and when converted to their 3,4-dihydro compounds and subjected to ultra-violet illumination, produce fluorescence of characteristic wavelength. This method has been widely used to demonstrate noradrenergic, serotoninergic and dopaminergic neurones in sections of the nervous system (eg. Kerkut et al., 1967).

In the absence of an equivalent technique for acetylcholine itself, attention has been directed towards the enzymes responsible for its synthesis and degradation. Staining for cholinesterase is not particularly useful in that the enzyme is not restricted to cholinergic neurone (Gombos & Aunis, 1982). Such staining has found its main application in the demonstration of motor end plates or of areas of general cholinergic activity (Koelle & Friedenwald, 1949; Karnovsky & Roots, 1964). The acetylcholine-synthesising enzyme, choline acetyltransferase, on the other hand, is generally accepted to be localised

within cholinergic neurones(Gombos & Aunis, 1982). A histochemical procedure for its determination was reported by Burt (1970). This method relies upon the precipitation of lead mercaptide by free Coenzyme A, generated in the synthesis of acetylcholine. The specificity of the technique was severely criticised by Hebb et al.(1970) and Kásа et al.(1970) who suggested that positive results depend in part upon the activity of other enzymes which liberate free Coenzyme A. They put forward an improved version of the technique which includes 10^{-3}M di-isopropylfluoro-phosphate (DFP). This reduces by 90% the non-specific hydrolysis of Acetyl-CoA, without affecting the activity of choline acetyltransferase(ChAT).

A modified method was used by Kim (1971) to demonstrate ChAT activity in chick spinal cord explants. In place of DFP, Kim used another specific inhibitor of acetylcholinesterase (BW 284 C51) to prevent the non-specific hydrolysis of Acetyl-CoA. He reported ChAT activity mainly in large motor neurones. The reaction was always negative if Acetyl-CoA and choline chloride were omitted.

In a critical analysis of the technique, Burt and Silver (1973) came to the conclusion that, even with all reasonable precautions, a maximum of 73% of the enzyme activity associated with the deposition of reaction product could be related to acetylcholine synthesis and hence ChAT activity. They discounted another compound, chloroacetylcholine (ClACh), used by Kasa et al. (1970) as not being "effective as an inhibitor".

In view of the lack of specificity of the above technique, it has now largely been discarded and superceded by an immunohistochemical approach (Section 1.2.8(i)).

Few other enzymes of neurotransmitter metabolism have been used in histochemical localisation. Detection has usually been with specific antisera or monoclonal antibodies (Section 1.2.8(i)), although detection of GABA transaminase, relies on its reduction of a tetrazolium salt. This method has been used to localise the enzyme activity in nuclei of rat and rabbit brain stem and spinal cord, and in rat cerebellar cortex (Gombos & Aunis, 1982).

1.2.5 RADIOLABELLED NEUROTRANSMITTERS AND PRECURSORS FOR THE IDENTIFICATION OF NEURAL ELEMENTS

A widely used method for the demonstration and localisation of specific neuronal tracts and cell types in neural tissue is through the use of radiolabelled neurotransmitters and/or their precursors. The neural tissue/culture under study is incubated with the labelled compound and the binding/uptake of the compound can then be demonstrated visually through the technique of autoradiography. Suitable controls (eg. in the presence of unlabelled compound) are performed to analyse the specificity of the binding/uptake.

Examples of the use of this technique include the demonstration of GABAergic neurones in dissociated cultures of human foetal spinal cord through the uptake of radiolabelled GABA (Touzeau & Kato, 1986; Kato et al., 1987). Apparently 60% of the neurones present were labelled by this method when the autoradiograms were subsequently developed and examined. As glial cells also take up GABA (for its catabolism) a specific inhibitor of non-neuronal GABA uptake had to be used, namely β - alanine. Another control was the inclusion of aminocyclo-hexane carboxylic acid (ACHC), which specifically blocks neuronal GABA uptake.

Thyrotropin-releasing hormone receptors have now been extensively mapped in the CNS through the technique of radio-ligand binding and autoradiography (Pazos et al., 1985; Manaker et al., 1985a; Manaker et al., 1985b). These studies have demonstrated high concentrations

of receptors in spinal cord so confirming the presence of TRH neuronal systems previously detected by immunohistochemical means (Sections 1.3.5(iii) and 1.2.8(i)).

An elegant technique which illustrates the versatility of autoradiography was published by Cuello et al., in 1980. They were able to label a monoclonal antibody to substance P directly and internally by growing the hybridoma cell line in radioactive medium. The combined use of this radiolabelled monoclonal antibody and a conventional antibody, raised in a different species, to enkephalin, was successful in revealing the coexistence of two neuropeptides in nerve terminals of the substantia gelatinosa. The monoclonal antibody was detected by autoradiography and the conventional antibody by peroxidase staining.

1.2.6 ASSAY OF SPECIFIC ENZYMES IN NEURAL TISSUE

In addition to the "direct visualisation" histochemical techniques for localising enzymes within neural tissue, there is also a need for assays of specific enzymes within the tissue (or culture) as a whole. This provides little indication as to the specific localisation of cells within a heterogeneous population, but is used to demonstrate the presence of cell-type specific enzymes and to assess levels of activity which may be related to the proportions of cells present.

The colorimetric method for the determination of acetylcholinesterase activity (Ellman et al., 1961) is still used as an indicator of the presence and activity of cholinergic cells (Digby et al., 1985).

A radiochemical method for the determination of ChAT activity (Fonnum, 1969; Fonnum, 1975), or one of its many subsequent modifications (eg. Berg & Fischbach, 1978) is, however, usually chosen to assay cholinergic activity, and hence the presence of cholinergic neurones in heterogeneous nerve cell preparations.

A number of enzymes are present to a much higher extent in glial cells than in neurones (eg. carbonic anhydrase, glutamine synthetase) and assays of these enzymes can be employed as "glial markers" (reviewed by Schousboe, 1982).

Three forms ($\alpha\alpha$,$\alpha\gamma$,$\gamma\gamma$) of the glycolytic enzyme, enolase, are found in the nervous system. The $\alpha\alpha$ form has been shown to be localised to glial cells, whereas the $\gamma\gamma$ form is confined strictly to neurones (Scarna

et al., 1982; Steinberg et al., 1983).

A sandwich enzymoimmunoassay has been successfully used to assess levels of the neurone-specific enolase (NSE) and hence to give an index of neuronal damage following experimental lesions of rat brain and also of lesions, following coma, in man (Scarna et al, 1982; Steinberg et al., 1984).

1.2.7 TOXINS AS SPECIFIC MARKERS OF NEURAL ELEMENTS

The first satisfactory marker specific for neurones was tetanus toxin. Mirsky and co-workers (1978) were able to demonstrate, by autoradiography and immunofluorescence, complete specificity of the toxin for cultured neurones from many different regions of the nervous system (brain, spinal cord, dorsal root ganglia etc.) of mouse, rat and chick. Subsequently, Raff et al. (1983) have claimed that Type II astrocytes also bind tetanus toxin. This emphasises the need for dual labelling in attempts to identify cell types unambiguously (eg. the additional use of anti-neurofilament antibody for the identification of neurones - see Table 4, p 70). The receptors for tetanus toxin have, until recently, been assumed to comprise solely the gangliosides, GD1b and GT1b. The binding studies which demonstrated this were, however, all carried out at non-physiological pH and salt concentration and now Critchley et al. (1986) have shown that "under more physiological conditions of salt and pH, the interaction of tetanus toxin with gangliosides is very weak and the membrane receptors for the toxin are protease-sensitive". They suggest that other membrane constituents such as sialoglycoproteins may be the true receptors for the toxin.

The cholera toxin B-subunit has been shown to recognise and specifically bind to the oligosaccharide chain of ganglioside, GM1. Doherty and Walsh (1987) have shown further that in dissociated dorsal root ganglion cells (from embryonic chick), the toxin binding appears

to be specifically to neurones. It would appear, however, that both GM1 and cholera toxin binding is more widespread in other culture systems or neural tissue (see Table 4, p 70) , which limits its usefulness as a specific neuronal marker.

It is possible that other toxins may be specific to neuronal sub - types. Botulinum toxin, for example, appears to be specific for cholinergic nerve terminals, if not for motor neurone terminals (Dolly et al., 1984).

Poliomyelitis virus is neurotropic and may bind specifically to motor neurones (Miller et al., 1980).

Young and Snyder (1973) provided evidence that strychnine binds to the putative glycine receptor of spinal cord synaptic membrane. Glycine is a major inhibitory transmitter within the spinal cord and the convulsions and muscular hyperactivity characteristic of strychnine poisoning would be consistent with its role as an antagonist. Strychnine affinity columns have been used to purify the glycine receptors. Further experimental data indicate that strychnine binding may be at an allosteric site, distinct from glycine binding, possibly the chloride ionophore (Pfeiffer & Betz, 1981).

Following the use of fluorescent-labelled anti-strychnine antibodies, it has been claimed (Bhattacharyya & Bhattacharyya, 1981; Srinivasan et al., 1983) that strychnine binding sites are restricted to spinal motor neurones. If this is indeed the case, then strychnine would constitue the only known direct marker for motor neurones to date.

1.2.8 ANTIBODIES FOR IMMUNOHISTOCHEMICAL LOCALISATION OF NEURAL ELEMENTS

1.2.8(i) ANTIBODIES RECOGNISING INTRACELLULAR ANTIGENS

Prime candidates for the production of marker antibodies are the proteins of the cell cytoskeleton (rev. Kalnins & Connolly, 1981; Cooper, 1986). The cytoskeleton comprises three groups of filaments: the microfilaments, intermediate filaments and microtubules (macrofilaments). The microfilaments and microtubules occur in most cell types and are composed of the proteins actin and tubulin respectively.

The intermediate (7-11nm) filaments are, however, largely composed of different protein subunits depending on cell type (summarised in Table 3, p 62). The presence of one type of intermediate filament protein in a cell does not, however, neccessarily exclude the presence of another. A particular example, as demonstrated by Shaw and Weber (1983), is the presence of vimentin in cells which are also positive for glial fibrillary acidic protein (GFAP).

Many polyclonal and monoclonal antibodies have been produced to intermediate filament proteins as cell-type specific markers or as probes to study their structure and function (Anderton et al., 1980; Wood & Anderton, 1981; Yen & Fields, 1981; Debus et al., 1982; Shaw & Weber, 1982; Lee et al., 1982; Eng, 1982; Goldstein et al., 1983; Sternberger & Sternberger, 1983; Trojanowski et al., 1986; Lee et al., 1986). Well characterised monoclonal antibodies to all the intermediate filament

Table 3 "Cell-type specific" intermediate filaments of the cytoskeleton

Cell type	I.F protein	M.Wts (approx)
Epithelial	cytokeratins	40KDa-70KDa
Mesenchymal eg.fibroblasts	vimentin	57KDa
Muscle	desmin	53KDa
Neurones	neurofilament protein	70KDa 160KDa 200KDa
*Glial cells	glial fibrillary acidic protein (± vimentin)	51KDa 57KDa

*Note Oligodendrocytes have no intermediate filaments- only microtubules

proteins are now commercially available.

Other prime intracellular candidates for specific antibody generation are neurotransmitter synthesising enzymes. In particular ChAT has received considerable attention. Early attempts to purify the enzyme, to raise monospecific antisera and apply the antibodies to staining techniques gave many problems (Malthe-Sorenssen et al., 1973; Eng et al., 1974; McGeer et al., 1974; Ryan and McClure, 1979; Peng et al., 1980) and the results were severely criticised (rev. Kása, 1986; rev. Cuello & Sofroniew , 1984).

Several groups have now reported the production and immunohistochemical use of monoclonal antibodies to ChAT (Levey et al., 1981; Eckenstein & Thoenen, 1982; Crawford et al., 1982;Nagai et al., 1983; Levey et al., 1983). In particular Barber et al. (1984) have published an elegant study of ChAT localisation, by a monoclonal antibody, in the rat spinal cord. The immunoreaction product was deposited in cell bodies, dendrites, axons and axon terminals, thus allowing comprehensive visualisation of entire cholinergic neurones and systems. The main findings were that ChAT-positive neurones are not restricted to the somatic motor neurones of the ventral horn and the pre-ganglionic autonomic neurones in sympathetic and parasympathetic spinal segments. Previously unknown ChAT-positive neurones were also identified in dorsal horn, central grey matter and in the intermediate spinal gray region. It was accordingly concluded that " cholinergic elements in the spinal cord are considerably more numerous and widespread than

was previously thought".

In a developmental study performed by the same workers (Phelps et al., 1984) it was shown that ChAT-positive cells in the intermediate region (named "partition" cells) are the most intensely stained and show immunoreactivity "earlier than any other putative cholinergic cells in spinal cord, including motoneurones".

These new findings must therefore bring into serious question the use of ChAT as a specific marker for motor neurones, especially in developing systems (including tissue culture).

Other enzymes of neurotransmitter metabolism which have been probed in the nervous system by immunohistochemical means include dopamine β-hydroxylase, tyrosine hydroxylase, dopa-decarboxylase, phenyl-ethanolamine-n-methyltransferase, (for the localisation of monoaminergic neurones) and glutamate decarboxylase (to map the neuronal pathways of the amino acid neurotransmitter gamma-amino-butyric acid (reviewed by Gombos & Aunis, 1982).

Antisera and monoclonal antibodies have also been produced to small neurotransmitter molecules by coupling them with a large immunogenic molecule (eg. BSA). Monoclonal antibody to serotonin was produced by Consolazione et al.(1981), and Geffard et al. (1984) have reported the first demonstration of highly specific and sensitive (polyclonal) antibodies against dopamine.

The neuropeptides are mostly highly immunogenic and many, including enkephalin and substance P have been localised to specific neuronal pathways by

immunohistochemical methods (Bloom, 1981). Worthy of a special mention in this respect is Thyrotropin-releasing hormone (TRH). TRH was isolated originally from hypothalamic tissue and was shown to stimulate the release of thyrotropin (TSH) from the anterior pituitary, both *in vivo* and *in vitro* (see Redding & Schally, 1969). Antibody to TRH was first prepared by Bassiri and Utiger (1972). TRH is only a tripeptide (1-pyroglutamyl-L-histidyl-L-prolineamide) and hence only weakly immunogenic. It was ,therefore, coupled to BSA prior to immunisation, which yielded highly specific and sensitive antisera.

Hökfelt *et al*. (1975) performed an immunohistochemical analysis of the distribution of TRH in the CNS and found that it was widespread, with TRH-staining fibers present even in rat spinal cord anterior horn. Sharif and Burt (1983), have demonstrated high affinity binding sites for [^{3}H]-TRH (or one of its analogues) in spinal cord.

The physiological role of TRH in the CNS (especially spinal cord) is reviewed in a later Section (1.3.5(iii)).

Acetylcholine, unfortunately, is not suitable for chemical conjugation in the same way as other small neurotransmitter molecules. To overcome this problem, Geffard *et al*. (1985a) synthesised acetylcholine-like conjugates from choline, glutaric anhydride and polypeptides in a manner such that the structure of acetylcholine was conserved. The antibodies produced were found to be highly specific for acetylcholine and were applied for the first time to immunocytochemical staining in

the locust brain (Geffard et al., 1985b). The wider application of this tool should greatly enhance our knowledge and understanding of cholinergic systems and acetylcholine subcellular localisation.

1.2.8(ii) ANTIBODIES RECOGNISING CELL-SURFACE ANTIGENS

Intracellular markers suffer from the disadvantage that the cells must be either disrupted, or fixed and permeabilised to allow access of the reagents (eg. monoclonal antibodies). The cells are therefore killed in the process. It is much more satisfactory to have a marker which will recognise a surface feature of a cell without causing it any damage. Such markers can also be used for the purification or selective depletion of specific cell types as previously mentioned (Section 1.1.4), and to demonstrate the variable expression of surface antigens on the same cell type during different stages of differentiation (Leibowitz & Hughes, 1983).

Cell surface antigens were first identified with adsorbed polyclonal antisera. A forerunner in this field was the Thy-1 antigen which was initially thought to be present only on thymocytes and thymus-derived lymphocytes. It was subsequently found, however, also to be present on fibroblasts. Moreover, after prolonged culture of nervous tissue, some astrocytes and neurones also become Thy-1^+. Oligodendrocytes, on the other hand, never express the Thy-1 antigen (reviewed by Schachner, 1982). A monoclonal antibody to Thy-1 antigen has been reported to stain not only the surface of a fibroblast cell line, but also vimentin of the intermediate filaments (Dulbecco et al., 1981).

The example of Thy-1, and of several subsequently-discovered antigens, emphasise the need for caution in describing a marker as "cell-type specific". It is

good practice to employ a series of markers so that cross reference can be made to categorise the cell types. There are now in existence many antisera, monoclonal antibodies and other markers which recognise cell-surface antigens in nervous tissue (Table 4, p 70) For example, Type I astrocytes are usually classified and identified as being $GFAP^+$, $A_2B_5^-$, Type II astrocytes as $GFAP^+$, $A_2B_5^+$, neurones as Tetanus $toxin^+$, $neurofilament\ protein^+$, (but $GFAP^-$) etc.

Athough there are a few reports of monoclonal antibodies which can distinguish between sub-populations of neurones from the CNS and PNS (Vulliamy et al., 1981; Cohen & Selvendran, 1981; Hirsch et al., 1983), there is still no definitive marker to distinguish motor neurones from other neurones and glia. This can, at present, only be achieved by the retrograde transport of a marker (usually horse-radish peroxidase) from the site of injection (skeletal muscle) to the cell bodies in the ventral horn (Neuhuber & Niederle, 1980). When applied to culture techniques it is necessary to kill the cells to develop the reaction after a few days in culture (Bennett et al., 1980). Alternatively, Smith et al. (1986) have used wheatgerm agglutinin coupled to Lucifer Yellow vinylsulfone in their foetal rat system. Unfortunately, this is only reliable for a few days after subsequent culture as the fluorescence diminishes.

A good example of a cell-surface marker which appears to be limited to a particular class of neurones is seen in the CHOL-1 antigen (Jones et al., 1981). The antiserum, raised to small synaptosomes prepared

from the purely cholinergic electric organ of the electric fish, Torpedo marmorata, appears to recognise cholinergic nerve terminals specifically. Recent evidence produced by Ferretti and Barroni (1986) suggests that the two gangliosides GT1b and GQ bear antigenic determinants specific for cholinergic neurones.

The diversity and immunogenicity of the gangliosides (reviewed by Ledeen, 1985) is perhaps the best hope for the identification of sub-sets of neurones. Kim et al. (1986) go as far as to suggest that "each neural cell type may contain a specific and characteristic set of gangliosides".

Table 4 Markers for Neural Tissue Components

(unless otherwise stated, markers are all antisera and/or monoclonal antibodies- see list of abbreviations, p vi)

Markers	Antigen	Surface or intracellular	Cellular specificities	References
β-S100	protein	IC	astrocytes	Cicero et al.,1970 Aono et al.,1988
GFAP	glial fibrillary acidic protein 51KDa	IC	astrocytes	Bignami & Dahl, 1973
FSA or LETS	glycoprotein	S	fibroblasts	Wartiovaara,1974 Hynes, 1976
RAN-1	trypsin sensitive	S	schwann cells	Brockes et al., 1977
GC	galactocerebroside	S	oligodendrocytes	Raff et al., 1978
γ-enolase	enzyme	IC	neurones	Schmechel et al., 1978 Scarna et al.,1982
α-enolase	enzyme	IC	glial cells	Schmechel et al., 1978 Scarna et al.,1982

continued

Table 4 (continued)

Markers	Antigen	Surface or intracellular	Cellular specificities	Reference
Tetanus toxin	gangliosides GDIb, GT1a and protein	S	neurones & sub-population type II astrocytes	Mirsky et al.,1978 Raff et al.,1983 Critchey et al.,1986
THY-1	glycoprotein 17.5KDa	S	fibroblasts also neurones & astrocytes (mature)	Raff et al.,1979
A_2B_5	ganglioside GQ1C	S	neurones,type II astrocytes oligodendrocytes (neurone specific in mouse retina)	Eisenbarth et al., 1979 Raff et al.,1979
*D2	polypeptide 139KDa	S	neurones	Jørgensen et al., 1980
38/D7	trypsin sensitive	S	PNS neurones	Vulliamy et al., 1981
A4	trypsin resistant	S	CNS neurones	Cohen & Selvendran 1981
RT97	neurofilament protein 200 & 160KDa	IC	neurones	Wood & Anderton, 1981. See also section 1.2.8(i)

continued

Table 4 (continued)

Markers	Antigen	Surface or Intracellular	Cellular specificities	Reference
ChAT	enzyme 66KDa	IC	cholinergic neurones	Levey et al.,1981 see also Section 1.2.8(i)
H-2		S	astrocytes,fibroblasts (cultured cerebellum)	Schnitzer & Schachner,1981
RAN-2	protease sensitive	S	astrocytes, ependymal cells müller cells,meningeal cells	Bartlett et al.,1981
NS-1	lipid	S	oligodendrocytes	Schachner,1982b
NS-2	polypeptides 84 & 120KDa & lipid	S	glioblastoma	Schachner, 1982b
NS-4	polypeptides 145 & 200KDa	S	neurones>>glia	Schachner,1982b
*BSP-2	glycoprotein 180,140 & 120KDa	S	neural cells	Rougon et al.,1982
BSP-3	glycoprotein 48KDa	S	astrocytes>>neurones	Hirn et al.,1982
F-10-44-2	glycoprotein 90KDa	S	white matter (glia?)	McKenzie et al.,1982

continued

Table 4 (continued)

Markers	Antigen	Surface or Intracellular	Cellular specificities	Reference
CHOL-1	ganglioside Gt1b & GQ	S	cholinergic neurones	Richardsonetal.,198 2 Ferretti & Borroni, 1986
*N-CAM	glycoprotein 140KDa, 170KDa	S	neurones	Choung et al.,1982 Rutishauser,1984
SSEA-1	glycolipid	S	astrocytes 80-90%	Lagenaur et al.,1982
°NILE	glycoprotein 200-230KDa	S	neurones	Salton et al.,1983 Sajovic et al.,1986
F3-87-8	glycoprotein 130, 100KDa	S	CNS specific	Lakin et al., 1983
MI/NI		S	astrocytes(80%)(human)	Dickson et al.,1983
308		S	astrocytes(80%)(human)	Dickson et al.,1983
01-04	glycolipids	S	oligodendrocytes (various developmental stages)	Schachner,1982
°LI	glycoprotein 40KDa,200KDa	S	neurones(cerebellar)	Rathjen & Schachner 1984
MRC OX-2	glycoprotein,41KDa	S	neurones (cerebellar)	Webb & Barclay,1984

continued

Table 4 (continued)

Markers	Antigen	Surface or Intracellular	Cellular specificities	Reference
SSEA-3 SSEA-4	glycolipid	S	sensory neurones (sub-set)	Dodd et al.,1984
224-58	glycolipid	S	Schwann cells	Guerci et al.,1986 Goujet-Zalc et al., 1986
F480		S	microglia (non-motile)	Austyn & Gordon,1981 Perry & Gordon,1988
C138	microtubules	IC	neurones (brain, sub-population)	Hirsch et al.,1983
Fibronectin		S	fibroblasts	Raff et al., 1979
MBP	myelin basic protein	S	myelinated fibre tracts	Jen Chou et al.,1986
M2		S	astrocytes, neurons, oligodendrocytes (immature)	Lagenaur & Schachner,1981
Cholera toxin	ganglioside GM1	S	neurones(80%)astrocytes(80%) oligodendrocytes,schwann cells (50-70%)fibroblasts(5-10%) (human). Neurones (DRG)	Kim et al.,1986 Doherty & Walsh, 1987
GQ	ganglioside	S	neurones(80-90%)astrocytes 10-20%)(human)	Kim et al.,1986

continued

Table 4 (continued)

Markers	Antigen	Surface or Intracellular	Cellular specificities	Reference
GM4	ganglioside	S	astrocytes(80-90%)oligo-dendrocytes,schwann cells (50%)(human)	Kim et al.,1986
GD3	ganglioside	S	astrocytes(5-10%)oligo-dendrocytes(human)	Kim et al.,1986
M1		IC	astrocytes(sub-population)	Schachner,1982
C1		IC	astrocytes(sub-population)	Schachner,1982
NG2		S	astrocytes, neurons	see Kim et al.,1986
S1		S	astrocytes, neurons	see Kim et al.,1986
RBO1 RBO2		S	oligodendrocytes	see Kim et al.,1986

* and ° indicate probable identical antigen (underlined)

1.3 INVESTIGATION OF MOTOR NEURONE DISEASE

1.3.1 INTRODUCTION

Neural tissue culture systems have been applied to many general studies of growth, development and differentiation, as already reviewed in the first two sections of this introduction.

In addition, dissociated neural tissue cultures are readily adapted to electrophysiological study. The mature cultures, usually in 35mm dishes, are mounted on the stage of an inverted phase contrast microscope modified to maintain temperature at 37°C. The bathing medium is either the culture medium, or a balanced salt solution at pH7.4. The morphologically identified neurones are large enough to be readily impaled with microelectrodes for intra-cellular readings. Drugs and other pharmacologically active substances can be applied in the vicinity of the neurone under study by iontophoresis. Studies of this nature have established resting membrane potentials, demonstrated the electrical excitability of neurones compared to glial cells and shown "normal" neurone-neurone synaptic interactions (rev. Nelson, 1975; Peacock et al., 1973; Ransom et al., 1977).

The responses of spinal neurones in culture to many drugs and biochemicals (including amino acids) have been recorded and in some cases a classification based on these data is possible eg. neurones from DRG will only respond to gamma aminobutyric acid (GABA), while spinal cord neurones will respond to most amino

acids (rev. Ransom & Barker, 1981).

Essentially, neurones in mature dissociated culture appear to behave in a similar manner to those in less disrupted systems previously studied (ie. in vivo, or organ culture).

The electrophysiology and pharmacology of neurone-muscle synapses in tissue culture has also been extensively studied with the use of combined cultures of neurones and muscle cells. Normally, a stable culture of myotubes is established first and neurones are added subsequently (Fischbach, 1972; rev. Nelson, 1975; Puro & Yeh, 1983).

Of particular interest in the field of neurone-muscle synaptogenesis in culture was the observation by Nurse and O'Lague (1975) that dissociated sympathetic SCG neurones from neonatal rats would form functional cholinergic synapses with rat myotubes in culture. α-Bungarotoxin blocked synaptic transmission from neurone to myotube, but not from neurone to neurone, thus indicating that the neuronal nicotinic ACh receptor might not be identical with the muscle nicotinic ACh receptor. Their data suggest the exercise of extreme caution in the classification of neurones which innervate skeletal muscle in culture.

Ransom and Barker (1981) summarised their review on the subject of the properties of mammalian central neurones in cell culture by suggesting that due to the "rich background information that has accumulated about the general properties of different (neural) culture systems", that they should be employed in a "more selective,

problem-solving" capacity in future. They envisaged that "a researcher will employ neural cultures when it is apparent that he can exploit certain of their unique features to answer his question most expeditiously".

The use of neural cultures as a "problem-solving" model system has been clearly recognised by workers interested in the pathogenesis and aetiology of Motor Neurone Disease (MND).

1.3.2 CLASSIFICATION OF MOTOR NEURONE DISEASE

Motor Neurone Disease is a collective term for a group of diseases characterised by initial degeneration of somatic α-motor neurone tracts, followed by muscular atrophy (assumed to result from the inability of the dead neurones to supply essential trophic factors to their target skeletal muscle - Section 1.1.6). There is, at present, no generally accepted cause for the degeneration of the neurones themselves.

The variations of the disease are classified (with much apparent ambiguity) according to the relative involvement of upper motor neurones and lower motor neurones and depending on the "segmental level" of the CNS at which the neurones are primarily affected.

The term Amyotrophic Lateral Sclerosis (ALS) is sometimes used interchangeably with MND (especially in the USA). It refers to the "hardening" of the spinal cord (produced by glial scar tissue) observed at the post-mortem investigation of a victim of MND (Rowland, 1984).

Clifford-Rose (1983) prefers to restrict the term ALS to those cases in which upper motor neurone signs predominate. Where lower motor neurone signs predominate, he uses the term progressive muscular atrophy (PMA). If the lower motor neurones of the lower cranial nerves are affected then swallowing, speech and facial muscles malfunction and this is termed chronic (progressive) bulbar palsy. Destruction of the upper motor neurones supplying the facial, tongue and throat muscles (following

a stroke for example), gives rise to similar symptoms in a condition termed pseudo-bulbar palsy.

In view of the confusion over terminology, the term MND will be used hereafter to cover all the variations of the disease. From the biochemist's point of view it is the term which best describes the central dysfunction ie. the death of motor neurones.

Not all somatic α-motor neurones are affected. In MND those supplying the eye muscles and the sphincter muscles of the bladder and anus are spared (Rowland, 1984). It is generally accepted also that the autonomic and sensory neurones are unaffected, except perhaps in some protracted cases (Steiner et al., 1984). Likewise, other neurones in the brain are unaffected and the intellect is unimpaired, although rare cases may be accompanied by dementia (Wilkstrom et al., 1982).

1.3.3 STRUCTURAL AND ULTRASTRUCTURAL PATHOLOGICAL CHANGES IN AFFECTED MOTOR NEURONES

At the structural level there is denervation, abnormal end-plates and some axonal sprouting in affected skeletal muscle (Bjornskov et al., 1984). The affected, but surviving, cell bodies appear shrunken and nuclear changes are common (Yates, 1977). A unique finding in MND is the occurence of amorphous material containing silicon which is deposited around, or embeds the endoplasmic reticulum and some cytoplasm. They are electron-dense and called intracytoplasmic inclusions (or Bunina bodies). Their significance, if any, is unclear (OKamoto et al., 1980).

The so-called typical central chromatolysis seen after axotomy is considered to be rare in MND. However, this has been reported to be abundant in one case of only 10 months duration (Hirano & Inoue, 1980). This response to axotomy is a relatively short lived reaction and probably does occur in the early stages of MND, but would not be seen in cases of longer progression (Sobue et al., 1983). The "signal" for chromatolysis is considered to result from the absence of a factor normally supplied by the periphery (Cragg, 1970).

1.3.4 INCIDENCE AND EPIDEMIOLOGY OF MOTOR NEURONE DISEASE

The incidence of the disease worldwide is 1-2 per 100,000. Males are affected more commonly than females (approximately 2:1). The mean age of onset is 66 years with an average duration of 3 years. Up to 10% of the cases are familial, the remainder are termed "sporadic", although they may occur in occasional clusters (eg. Melmed and Krieger, 1982).

Some studies (reviewed by Kurtzke, 1982) have shown an increased incidence of the disease associated with previous trauma to the limbs, or in occupations where heavy labour is involved (especially pneumatic drilling and farming). Buckley et al.(1983) have reported that in England and Wales (1959-1979) the greatest occupational excess of MND sufferers was in the leather industry. Whether this was associated with the labour involved, a chemical toxin used in the process (such as a dye substance) or some other factor, is unknown.

The disease used to occur at a much higher frequency (50x in 1954) in certain islands of the western pacific: notably Guam, West New Guinea and the Kii peninsula of Japan (Nemo et al., 1974; Hoffman et al., 1981; Garruto et al., 1981; Yanagihara et al., 1983). This "Guamanian" form of MND used to account for up to 20% of all deaths in the regions affected. The onset of the disease was earlier than the sporadic form and was commonly accompanied by Parkinsonian-Dementia (PD). There is a major pathological difference between the "Guamanian" and "sporadic" forms of MND. On post-mortem examination of "Guamanian" disease victims it is found that affected upper and lower motor

neurones have "neurofibrillary tangles" ie. the normal structure of the axonal cytoskeleton appears to have been disturbed, resulting in abnormal accumulation and morphology of the axonal filaments (Garruto and Yase, 1986). Interestingly, this is also seen in the brains of those patients who have died with Alzheimers disease (see Appel, 1981), but only rarely in cases of "sporadic" MND.

Garruto et al. (1985) have reported that the incidence of MND and P-D on Guam and the other islands has declined steadily until it is now only slightly higher than the sporadic incidence. This remarkable occurence seems to be linked with the increasing "civilisation" of the islanders, and strongly suggests the reduction in an environmentally available "trigger" for the disease.

In their most recent review of the data, Garruto and Yase (1986) rule out several possibilities for the source of the neurotoxic agent, such as the ingestion of a cyanogenetic glycoside (cycadin) present in the locally distributed cycad nut. They concentrated on the mineral composition of the local soil and drinking water. These are both unusually low in calcium and magnesium and relatively rich in aluminium and iron in all three foci. Moreover, patients show deposition of calcium and aluminium in affected neurones, and disturbances in calcium and vitamin D metabolism. Garruto and Yase (1986) concluded that-"The accumulating epidemiological, genetic and environmental evidence,

as well as the experimental results increasingly support the hypothesis that a basic defect in mineral metabolism and secondary hyperparathyroidism, provoked by chronic nutritional deficiencies of calcium and magnesium, led to increased intestinal absorption of toxic metals and deposition of calcium, aluminium and other metals in neurones of patients with ALS and P-D".

That elemental deposition can lead to the production of neurofibrillary tangles has been confirmed experimentally in animals with aluminium intoxication (Selkoe et al., 1979). It seems likely that the accumulation of neurfibrillary tangles interferes with the normal functioning of the nerve cells, leading to their death (Section 4.1.6).

Deary and Powell (1986) have, however, suggested that care should be excercised in the interpretation of the mineral spectra obtained from the postsynaptic densities prepared from the brains of Guamanian ALS patients (and from those with Alzheimers disease and Parkinsonian-dementia). They have shown nearly identical spectra from the cerebral cortex of human foetuses, and suggest that the mineral deposition may be secondary - a result of the concentration of neuronal elements.

Most recently (Spencer et al., 1987), attention has returned to the ingestion of the seed of the plant Cycas circinalis l. as the potential source of environmentally available neurotoxin. Macaque monkeys were fed long term with β-N-methylamino-L-alanine (L-BMAA), which is present in the cycad nut. The animals developed

"corticomotoneuronal dysfunction, parkinsonian features and behavioural anomalies, with chromatolytic and degenerative changes of motor neurons in cerebral cortex and spinal cord". The cycad plant is now only rarely used as a food source in Guam, and this is consistent with the observed decline in incidence of Motor Neurone Disease and Parkinsonian-dementia on the island (Garruto et al., 1985). There is also a precedent for these findings in that it is accepted that human lathyrism (an upper-motor neurone degenerative disease) results from excessive ingestion of the chickling pea Lathyrus sativus. This species contains the accutely neurotoxic amino acid β-N-oxalylamino-L-alanine (BOAA), which may manifest its activity through its role as a potent glutamate receptor agonist (Spencer et al., 1984). In view of these findings it may well be that Guamanian MND-PD will be referred to as "cycadism".

The above data are consistent with the "excitotoxin" concept of Olney (see Coyle, 1982) who proposed that acidic amino acids, which have potent neuroexcitatory effects, cause neuronal degeneration by excessively depolarising neurones via. specific receptors responsive to these amino acids.

1.3.5 MAIN HYPOTHESES OF THE AETIOLOGY AND PATHOGENESIS OF SPORADIC MND

In sporadic MND, neurofibrillary tangles are rarely seen. Many possible causes of the disease have been cited and examined experimentally.

A major problem is that many demonstrated abnormalities can be interpreted as secondary effects rather than primary causes. In particular, there are many reports of impairment in neurotransmission, and alterations in levels of neurotransmitters and/or their synthesising enzymes and receptors in MND (Denys and Norris, 1979; Hayashi et al., 1981; Festoff and Fernandez, 1981; Belenduik et al., 1981; Gillberg et al., 1982; Whitehouse et al., 1983; Manaker et al., 1985; Gillberg and Aquilonius, 1985).These are, most probably, all secondary features related to the loss of neurones and denervation in MND. Anticholinesterase drugs may occasionally relieve muscle fatiguability, but they do not result in long term clinical improvement (see Fowler, 1984).

Other MND-related abnormalities, which are likely to be secondary in nature, include increased amino acid levels in motor cortex (Yoshimo et al., 1979), elevated CSF protein (Guiloff et al., 1980), abnormal carbohydrate metabolism (Saffer et al., 1977) and abnormal calcium metabolism (Mallette et al., 1977).

Some of the possible primary causes of MND that have recently received serious attention will be discussed in detail below.

1.3.5(i) Persistent or Previous Viral Infection

One hypothesis, now sharply declining in popularity, is that MND is caused by persistent or previous neurotropic viral infection (Matthews et al., 1977; Pertschuk et al., 1977; Kott et al., 1979; Weiner et al., 1980; Miller et al., 1981). It is still accepted however, that there is an elevated risk of developing MND following early infection with polio virus. Nevertheless, this has now been eliminated from the population and yet sporadic MND continues unabated.

There has recently been a small upsurge in the belief that at least some cases of MND may be the result of viral infection (reviewed by Rowland, 1987). For example, the human immunodeficiency virus (HIV) responsible for the acquired immunodeficiency syndrome (AIDS) can penetrate spinal cord and peripheral nerves and a young patient has been seen with both AIDS and typical ALS.

1.3.5(ii) Deficient Repair of DNA Damage

Another hypothesis which remains to be proved or disproved is that MND results from abnormalities in neuronal DNA and its function. In particular, there may be a deficiency in its repair mechanisms (Davidson and Hartmann, 1981; Bradley & Krasin, 1982; Unger et al., 1985). This hypothesis does not explain the specificity for motor neurones.

1.3.5(iii) Deficiency of Thyrotropin Releasing Hormone

As reviewed earlier (Section 1.2.5, 1.2.8(i)),

Thyrotropin-releasing hormone is widespread in the CNS. The accumulating evidence suggests that it acts as a neuropeptide with modulatory activity on other neurotransmitter systems. The cholinergic system has been specifically implicated in this respect, as it has been shown that TRH potentiates the excitatory actions of cholinergic agents on neurones (see Pazos et al., 1985). This activity could well explain the transient improvement seen in patients with ALS who were infused with TRH (Engel et al., 1983a).

Reduced levels of TRH in the cerebrospinal fluid of patients with MND (Engel et al., 1983b) and lower concentrations of TRH receptors in brain tissue (Mitsuma et al., 1986) and the spinal cord (Manaker et al., 1985a) have been reported, but these may well be secondary phenomena.

In view of the clinical improvement seen by Engel et al.(1983), however, a number of other trials have been undertaken with TRH (Imoto et al., 1984) or one of its longer acting analogues (eg. Guiloff et al., 1987). This therapeutic approach may prove useful in boosting the performance of surviving neurones, but it seems unlikely to be effective in halting the progressive course of neuronal degeneration. A longer term neuronotrophic activity has, however, been proposed for TRH, largely through its reported activity to boost ChAT activity and stimulate neurite outgrowth in cultured rat ventral spinal cord (Schmidt-Achert et al., 1984). It has also been reported to reverse the neuronal damage

caused by the substance P antagonist, spantide, and to stimulate myelin lipid synthesis in chick neural cultures (see Griffiths, 1986).

Recently, Court et al. (1987) reported an increase in the level of thyrotropin releasing hormone in the wobbler mouse (Section 1.3.6), but no changes in three patients with motor neurone disease.

1.3.5(iv) Autoantibodies or other Toxic Blood Borne Factors

This hypothesis is perhaps one of the most amenable to testing with neural tissue culture as a model system. It concerns the possibility that there may be present in the body fluids of patients with MND a component of exogeneous or endogeneous origin which is specifically toxic to motor neurones.

A key finding in this respect was the report by Wolfgram and Myers (1973) that 70% of diluted sera from patients with MND are toxic to the anterior horn cells of the neonatal mouse spinal cord in tissue culture, as assessed by morphological criteria. Sera from other neurodegenerative diseases, and controls, were reportedly inactive in this respect. The toxic factor was non-dialysable and hence presumed to be protein in nature. The toxic factor was also proclaimed to be different from that previously reported by Bornstein and Appel (1965) and Field and Hughes (1965). The latter was found to be present in 60% of ALS serum and to cause dymelination of cultured mouse cerebellar cells. This

factor had been shown to be antibody in nature. Its presence in a high proportion of ALS sera is almost certainly a secondary occurrence as there is secondary demyelination in MND.

The original finding of Wolfgram and Myers could not, however, be repeated by Horwich et al. (1974) "using the same technique as the previous investigator". Neither could it be confirmed by Liveson et al. (1975) using organotypic nerve and muscle tissue cultures, or by Ecob et al. (1984). Nevertheless, Roisen et al. (1982a,b) reported the demonstration of an "unique, highly specific, non-dialysable, heat labile, apparently complement-independent antineuronal factor in the sera of about 75% of all ALS patients". The factor was "neither rapid or long acting"; it required considerable time (5-6 days) to act". The culture system used was like that used by Wolfgram and Myers: explanted neonatal mouse spinal cord sections, and the cytotoxic activity was reported to be ineffective when tested on embryonic neurones. More recently, Maher et al. (1987) have also confirmed the finding of some cytotoxic activity of ALS sera for cultured spinal neurones.

Askanas et al. (1981) tested CSF from patients with MND and other neuromuscular disorders on explants of embryonic rat spinal cord. They saw no alteration in morphology of the cultures, neither was the level of neurone specific enolase (NSE) affected.

Touzeau and Kato have investigated the effects of ALS serum incorporated in the media of dissociated

neuronal cultures from both the ciliary ganglia of chick embryo's (1983) and human foetal spinal cord (1985). They found no differences in neuronal survival, ChAT activity, glutamic acid decarboxylase (GAD) activity and lactate dehydrogenase (LDH) activity after lengthy periods in culture, when compared to neurones grown in control sera. It is important to note, however, that unlike previous studies, the sera were heat inactivated before inclusion in the media. This would destroy the effectiveness of a complement-dependent cytotoxic antibody, or of other heat-sensitive, directly acting neuro-cytotoxic proteins (eg. enzymes).

Ronnevi et al. (1984), and Conradi and Ronnevi (1987) have recently described an antibody mediated destruction of normal erythrocytes when incubated with serum from patients with MND, when compared to controls. Other neurological disease (OND) sera have much lower levels of this cytotoxic activity. The relationship of this phenomenom, if any, to neurone damage is unclear.

Digby et al. (1985), using an alternative approach, showed significantly increased binding of serum immunoglobulins from MND patients to live, mature cultures of embryonic rat spinal cord cells when compared to control and OND sera.

The possibility that MND may have an immune system involvement has also been studied in other approaches. Tissue typing studies have shown increased frequencies of HLA types A3 and B12 (Antel et al., 1979; Antel et al., 1982), A2 and A28 (Behan et al., 1976) and BW35

(Kott et al., 1976) in MND. Other work, however, gave no evidence of differences in frequency from controls (Pedersen et al., 1977) and one study showed a decrease in HLA-A9 (Bartfeld et al., 1982).

The concentrations of immunoglobulins and complement factors in the sera of ALS patients are generally reported to be normal (Whitaker et al., 1973; Tavolato et al., 1975; rev. Antel et al., 1979). The occasional report of elevated levels of immunoglobulins eg. in Guamanian MND (Hoffman et al., 1981) is put down to secondary infections rather than to a specific anti-viral or autoimmune response.

Plasmapheresis with or without immunosuppression has had no effect on the course of the disease (Silani et al., 1980; Keleman et al., 1983). There is no inflammation or lymphocytic infiltration of neural tissue in the disease (Tavolato et al., 1975).

A few studies have detected immune complexes in neural biopsy tissue and sera of MND patients (Norris, 1979; Oldstone, 1976; Noronha et al., 1978). Others have reported negative findings (Barron et al., 1978; Antel et al., 1979; Rissanen, 1978). It is, once again, most likely that positive findings in this respect are a consequence of the disease rather than its cause.

The possibility of immune cellular abnormalities in MND patients have also been studied with mixed success. Kott et al. (1976), Behan (1979), Hoffman et al. (1978) all reported depressed or negative skin tests to common antigens. The in vitro lymphocyte proliferative response

to non-specific mitogens has been reported to be both normal (rev. Antel et al., 1979) and abnormally low (Hoffman et al., 1978; Behan et al., 1979; Digby et al., 1985; Aspin et al., 1986).

Nemo et al. (1974) found no difference in the response of lymphocytes from Guamanian ALS patients to specific brain antigens compared to controls. With lymphocytes separated from the blood of patients with sporadic MND, Aspin et al. (1986) showed a proliferative response to rat spinal cord cell membranes in 4 out of 14 cases as compared to 0 (from 9) in controls.

Bartfeld et al. (1985) found no significant changes in the percentages of immunoregulatory and activated T cells in ALS patients.

The significance of the increased binding of the serum immunoglobulins from MND patients to rat spinal cord cells in culture (Digby et al., 1985) and other positive immune system findings (Aspin et al., 1986) is discussed (Section 4.1.6i) with reference to additional data on this subject presented in the Results section (Section 3.1.10).

The hypothesis that immune system dysfunction is a causative and/or contributory factor in MND overlaps to some extent with other theories. One of these, the "viral theory" has already been mentioned (Section 1.3.5(i)). It is also possible that viral antigens induce antibodies which cross-react with some of the host's antigens. In this context, Fujinami et al. (1983) used monoclonal antibodies to demonstrate common antigenic determinants on measles virus phosphoprotein, herpes

simplex virus protein, and a human intermediate filament (probably vimentin). This "molecular mimickry" may be of significance in the appearance of so-called auto-antibodies during virus infection, although whether or not they are themselves involved in the disease process is unclear.

Another potential variation of autoimmune disorder may be associated with paraproteinaemia. It has been found that around 5-7% of cases in a large group of patients with MND had monoclonal immunoglobulin production. Moreover, it was demonstrated that in at least two examples, the immunoglobulin produced was directed against neuronal elements, the antigen being gangliosidic in nature (Freddo et al., 1987; reviewed by Rowland, 1987). The disease in these individuals is restricted to the lower motor neurones and hence is similar to the rare form of MND (5% of cases called pure progressive muscular atrophy (PMA). The site of autoimmune attack may well be at the exposed periphery ie. synaptic connections. Upper motor neurones are presumably unaffected because of their so-called "immunological privilege" (see Lampson, 1987).

1.3.5(v) Deficiency of Specific Neuronotrophic Factors

Involvement of the immune system has also been implicated in the next hypothesis of the aetiology and pathogenesis of MND. If, as has been suggested by much data (reviewed in Section 1.1.7), mature motor neurones rely upon a continuous supply of a specific

trophic factor from their target cells, then a deficit in this factor would result in premature death of motor neurones. It has already been shown that inactivation or destruction of NGF by a specific antisera has devastating effects on the viability of sympathetic and sensory neurones in the developing chick (Cohen, 1960) and that NGF is essential to support mature sensory neurones (Johnson & Yip, 1985). Hence, by analogy, it has been proposed that, if antibodies to the equivalent neuronotrophic factor(s) were present in adult humans, this would lead to death of motor neurones.

Gurney et al. (1984a; 1984b) mimicked denervation in mouse gluteus maximus muscle by the administration of botulinum toxin which blocks acetylcholine release from motor nerve terminals. They found that the terminal and collateral sprouting that this elicits was reduced when the muscle was treated with sera from 9 out of 19 sporadic ALS patients and 2 of 6 with familial ALS, but not with control or OND sera. Furthermore, on immunoblot analysis, the ALS sera recognised a 56KDa protein secreted by denervated rat diaphragm muscle. An antiserum raised against this protein also suppressed terminal axonal sprouting in the mouse botulinum-treated gluteus maximus muscle. Unfortunately, these dramatic findings could not be confirmed in similar studies by Hauser et al. (1986) and Ingvar-Maeder et al. (1986). Nor were Donaghy and Duchen (1986) able to demonstrate the inhibition of experimentally induced sprouting with sera from patients with MND and associated paraproteinaemia.

In immunoblot studies, Jehanli et al. (1986), reported more common binding of serum immunoglobulins from patients with MND to the three neurofilament polypeptides (Mr 200, 150 and 70KDa) prepared from rat spinal cord. Antibodies to the 150KDachain predominated. Brown et al. (1987) failed to show any consistent differences in immunoblot studies with sera from patients with MND or controls directed against spinal cord homogenates. They did, however, report that staining to 52KDa and 70KDa proteins was more common. The implication, from immunohistochemical evidence, was that the 70KDa antigen may have been the low molecular weight neurofilament polypeptide. The 52KDa antigen is very close to the molecular weight of glial fibrillary acidic protein (51KDa). Antibodies directed towards cytoskeletal elements are unlikely to be significant in the aetiology of MND.

In connection with the above reports, data have recently been published (Henderson et al., 1987) which demonstrate inhibition of neurite-outgrowth promoting activity by muscle extracts (15 out of 20) from patients with spinal muscular atrophy (SMA). This is an inherited disorder of early onset in which only the lower motor neurones degenerate, making it similar to "pure" progressive muscular atrophy (PMA), the rarest form of motor neurone disease (Section 1.3.2). The inhibition of neurite outgrowth from dissociated chick spinal neurones is apparently specific to that evoked by neonatal chick skeletal muscle extract. There is no effect on the neurite outgrowth promoting activity of embryonic chick skeletal muscle

extract. The inhibition is maximal at 1-10μg/ml of SMA extract. Above this dose, the human muscle extract itself begins to show neurite-outgrowth promoting activity. There was no inhibitory activity in an equal number of age matched pathologic or morphologically normal controls.

The above results raise the possibility that there may be inhibitors of motor neurone growth factors present in muscle from patients with SMA. The nature of these factors and their significance is not clear.

All of the above reports have concentrated on one aspect of neuronotrophic activity only; namely neurite outgrowth promoting activity. It has been shown that this activity can reside in a different molecule from the cholinergic stimulatory activity (Smith et al., 1985). It is not clear whether the survival promoting activity of some conditioned media or tissue extracts is associated with a separate molecule, or either or both of the others. It may be a case of degree ie. a minimal amount of a neuronotrophic factor maintains cell survival, while larger quantities have stimulatory activities. The demonstration of inhibitors (or destroyers) of a motor neurone survival factor would have the greatest implications in Motor Neurone Disease.

There are many other antecedent events which could lead to the reception by the motor neurone soma of an inadequate supply of life-sustaining trophic factors. It is possible that there may be antibodies to the receptor for the trophic factor(s) on the motor neurone

terminal membrane. Hence, even if a normal supply of trophic factor(s) were available for the muscle fibre, its uptake might be blocked, or the receptors destroyed, thus resulting in insufficient factor reaching the soma via fast retrograde axonal transport.

Alternatively, the muscle fibres may just stop synthesising or secreting the factor for some unknown reason.

A third possibility is that the retrograde transport of the factor may be interrupted. This is a distinct possibility in Guamanian MND where neurofibrillary changes are seen (Garruto & Yase, 1986).

Using the technique of video-enhanced differential interference contrast optics and computer analysis (see Schnapp & Reese, 1986), Breuer et al. (1986) have revealed abnormalities of fast retrograde axonal transport in excisionalbiopsies of motor nerve from patients with advanced sporadic MND. The retrograde organelle traffic density was reduced by 68.4% compared to controls, with a combined reduction in the speed of their movement. Anterograde transport was essentially normal, as were the ultrastructural features of the axons. A reduction of this nature could result in an insufficient supply of trophic factor(s) to the soma and lead to cell death.

Appel (1981) extended the "trophic factor" hypothesis of the aetiology of MND to include Parkinsons disease and Alzheimers disease. These are neurodegenerative diseases similar to MND, in which specific sets of neurones are affected, usually in later life. In the "unifying hypothesis" proposed, these diseases would all be due

to the lack of a specific neuronotrophic factor for each set of neurones. This notion has been picked up recently by Korsching (1986) who speculates that Alzheimers dementia may be due to the abnormal synthesis or utilisation of a trophic molecule (perhaps NGF) by the magnocellular cholinergic neurones affected in the disease.

It seems likely, as suggested again recently by Rowland (1987), that there is no single causative factor in Motor Neurone Diseases, but they can be triggered by many different antecedent events. The specificity of the disease does, however, point to the induction of a common biochemical disorder which results in the death of the neurones. An attractive possibility for this mechanism would be through interference with a specific motor neurone survival factor. Positive immune system findings, defective DNA repair, decreases in receptors, neurotransmitters and enzymes etc. would all follow as secondary and, perhaps, amplifying effects. Muscular atrophy in turn would follow due to the failure of the dead nerve cells to supply a trophic factor to the muscle (Section 1.1.6).

A particular attraction in the study of motor neuronotrophic factors is that this might lead to a rational therapeutic approach in Motor Neurone Disease. Even if the triggering events were not identifiable or preventable, or the common biochemical disorder is not the induced deficiency of a motor neurone survival factor, it is still possible that exogeneous administration of such a factor could slow or halt the progress of

the disease and even stimulate or allow for some peripheral re-innervation to occur. There is a precedent for this in that NGF has been shown to rescue neurones otherwise doomed to die through axotomy or colchicine administration (Section 1.1.7).

1.3.5(vi) Environmentally Available Neurotoxins

It has long been suspected that exposure to heavy metals (especially lead and mercury) may be antecedent events in some cases of MND (reviewed by Yanagihara, 1982). Accumulating evidence now strongly suggests that at least some cases of sporadic MND may result from exposure to specific neurotoxins available in the environment. In particular, the recent evidence concerning the involvement of a plant neurotoxin in Guamanian MND-PD (Spencer et al., 1987) is persuasive in the light of the link between an upper motor neurone disease (lathyrism) and consumption of another plant neurotoxin (Spencer et al., 1984). In addition, the example of MPTP (1,2,5,6-methylphenyltetrahydropyridine) which induces Parkinsons disease in animals and humans (reviewed by Langston, 1985) demonstrates that neurotoxicity specific to a limited set of neurones (dopaminergic nigrostriatal neurones) is possible. The active neurotoxic compound is, in fact, MPP^+ which is produced from MPTP in the body through the actions of the enzyme monoamine oxidase (MAO). MAO inhibitors have been found to be protective against the induction of Parkinsonism produced through MPTP administration. In addition, there is a lower incidence

of Parkinsonism in patients taking MAO inhibitors regularly to alleviate depression (Langston, 1985; Snyder & D'amato,1986).

MPP^+ was apparently used in the 1960s as a herbicide, especially in an agricultural area of Quebec. There is a higher incidence of Parkinsonism in this area, especially among farmers (Horizon,1987). Interestingly, there is also a higher incidence of MND among farmers (Buckley et al., 1983) and it is possible that this is caused by exposure to a selective neurotoxin present in agrochemicals, rather than heavy labour as was originally supposed.

It is possible that a single exposure to a neurotoxin anytime during the patients lifespan would be enough to induce neurodegenerative disease symptoms in later life. In the case of Parkinsonism, symptoms only develop when greater than 80% of the dopaminergic neurones have degenerated. Neurotoxic reduction of the number of neurones from, say, 80% to 50% would therefore not induce Parkinsonism until ageing and other general neurotoxic stimuli had further reduced the levels to 20% (see Langston, 1985).

1.3.6 ANIMAL MODELS AND THERAPEUTIC APPROACHES

There are two well-known animal models of motor neurone disease: hereditary motor neurone disease in the so-called "wobbler" mouse (Harris, 1975) and hereditary canine spinal muscular atrophy (Cork et al., 1980). Apart from basic morphological and physiological studies, few experiments have bee reported using these animals. In 1983, however, Lange et al., performed a therapeutic trial on wobbler mice in which gangliosides (previously reported to enhance neuronal regeneration and sprouting) and thymosin (reportedly effective in induction and maintenance of immune function) were administered long term. There was no improvement in either group.

Clinical trials have also been carried out with ALS patients being given daily injections of gangliosides over several months (Bradley et al., 1984; Harrington et al., 1984). No significant improvement was reported in either case.

It has recently been claimed that a syndrome similar to progressive muscular atrophy has been induced in guinea pigs through the repeated immunisation of highly purified swine spinal motor neurones (Engelhardt & Joo, 1986). The observed destruction of lower motor neurones in the guinea pigs appeared to be immunologically mediated and suggests common antigenic determinants with swine motor neurones. The site of induced cross-reactive autoimmune attack may have been at the periphery (ie. synapses) or, perhaps, through retrograde transport to the perikaryon (see Rowland, 1987). This animal model

may prove to be a useful tool to test therapeutic approaches to MND, although it may only be applicable to those rare cases of pure progressive muscular atrophy, and even then, only if they themselves are produced through an immunological mechanism.

Experimental Amyotrophic Lateral Sclerosis can also be induced in the guinea pig through chronic vitamin C withdrawal (den Hortog Jager, 1985). This can be prevented by administration of n-acetylcysteine and dithiothreitol. In clinical trials, de Jong et al. (1987) reported stabilisation in 26 out of 42 MND patients receiving these drugs for 6 months. Küther and Struppler (1987), however, could find no such improvement in patients given n-acetylcysteine alone.

other attempts at therapy have largely centred around the administration of anticholinesterase drugs (Section 1.3.5) and Thyrotropin Releasing Hormone (Section 1.3.5(iii).

AIMS OF THE PROJECT

1. To investigate additional factors required for the improved growth and survival of spinal cord cell cultures grown in serum free medium (SFM).

2. (i) To develop a protocol for the successful growth of human foetal spinal neurones in culture with the emphasis on maintenance in serum free medium (SFM).

(ii) To use the human cultures in comparative studies with rat cultures (with respect to growth, development and disease processes).

3. To carry out further studies into Motor Neurone Disease of particular relevance to the autoimmune/ neurocytotoxic factor hypotheses through the investigation of serum immunoglobulin binding to spinal cord cell cultures.

4. The production of an anti-neurofilament monoclonal antibody for the routine identification of cholinergic neurones in spinal cord cell cultures.

5. The production of an anti-choline acetyltransferase monoclonal antibody for the identification of cholinergic neurones in spinal cord cell cultures.

6. The production of (potentially novel) anti-neuronal cell surface monoclonal antibodies for
i) identification of neurones in culture
ii) purification of neurones from heterogeneous cell populations and/or selective depletion from spinal cord cell cultures through complement-mediated destruction

6. iii) study of novel neuronal cell surface antigens (eg. during development of cultures.

7. The production of other monoclonal antibodies, with cell-type specificities, for the further characterisation of the spinal cord cell cultures.

2. MATERIALS AND METHODS

2.1 MATERIALS

Standard plastic tissue culture treated dishes, multiwell trays and flasks were obtained either from Flow laboratories (Irvine, Scotland, U.K.) or GIBCO BRL (Paisley, Scotland, U.K.) . Culture medium (DMEM and RPMI 1640), supplements (glutamine and penicillin-streptomycin) and Foetal Calf Serum (FCS) were also from either of the above two suppliers. Batch tested Donor Horse Serum (DHS) was obtained from Northumbria Biologicals Ltd. (Cramlington,Northumberland, U.K.). Accessory tissue culture items (Petri-dishes, pipettes and tubes) were ordered from Walter Sarstedt Ltd. (Beaumont Legs, Leicester, U.K.), Sterilin Products Ltd. (Teddington, Middlesex, U.K.) or Atom Medical Supplies (Hove, Sussex, U.K.).

Glass coverslips (13mm dia.) were obtained through BDH Chemicals Ltd. (Poole, Dorset, U.K.) and PTFE-coated multitest slides (10 well x 8mm dia.) were from Flow Laboratories (Irvine, Scotland, U.K.).

All general chemicals were from BDH Chemicals Ltd (Poole, Dorset, U.K.) or Fisons (Crawley, Sussex, U.K.). Biochemicals and immunochemicals were from Sigma Chemical Company (Poole, Dorset, U.K.) unless otherwise stated.

[^{14}C] or [^{3}H] Acetylcoenzyme-A was obtained from Amersham International (Amersham, U.K.) at a specific activity of 1-5Ci/mmole.

Nitrocellulose paper was from Millipore (Bedford, U.K.) or Sartorius (Belmont, Surrey, U.K.).

Filters for sterilisation of heat-sensitive liquids (0.22μm) were obtained through Sera Lab Ltd. (Crawley, Sussex, U.K.).

Pregnant Wistar rats were bred and supplied by the animal house, Bath University (Bath, Avon, U.K.).

Foetal and adult human tissue was collected from local hospitals in Bath and Bristol, with the approval of the Ethical Committees.

Tetanus toxoid was a generous gift from Dr. Jonathan Lamb of University College Hospital, London, U.K. The tetanus toxin anti-sera was prepared in the department by Dr. J. Nickless.

The X-63 8Ag mouse myeloma cell line was originally purchased from Flow Laboratories (Irvine, Scotland, U.K.).

Guinea pig complement was from Miles laboratories (High Wycombe, Buckinghamshire, U.K.) or Sera Lab Ltd. (Crawley, Sussex, U.K.).

2.2 MEDIA AND SOLUTIONS

The compositions of important media and solutions are listed below.

PUCKS BALANCED SALT SOLUTION (Ca^{++} and Mg^{++} free) pH7.3

NaCl	137mM	GLUCOSE	33mM
KCl	5.4mM	SUCROSE	44mM
Na_2HPO_4	0.16mM	HEPES	10mM
KH_2PO_4	0.22mM		

SERUM SUPPLEMENTED MEDIUM (SSM) pH7.4

The basic medium was Dulbecco's Modification of Eagles Medium (DMEM) containing sodium bicarbonate (0.85g/l) and glucose (4.5g/l) to which the following were added:

Foetal Calf Serum (FCS)	10%
Donor Horse Serum (DHS)	10%
Penicillin/Streptomycin (PEN/STREP)	100U/ml
Glutamine	2mM
Glucose	1.5g/l

SERUM FREE MEDIUM (SFM) pH7.4

The basic medium was DMEM, as above, to which the following were added:

INSULIN	0.2µg/ml	PROGESTERONE	$2x10^{-8}M$
BIOTIN	1µg/ml	SODIUM SELENITE	$3x10^{-8}M$
THYROXINE	3µg/ml	PUTRESCEIN	$1x10^{-4}M$
TRANSFERRIN	5µg/ml	HYDROCORTISONE	$5x10^{-7}M$
PEN/STREP	100U/ml	GLUTAMINE	2mM
GLUCOSE	1.5g/l		

HEPES BUFFERED EAGLES MEDIUM (H-EAGLES) pH7.4

The basic medium was Eagles Minimum Essential Medium (MEM) to which the following were added:

$NaHCO_3$	0.85g/l
HEPES	20mM
GLUTAMINE	2mM
PEN/STREP	100U/ml

CLONING MEDIUM (CM)pH7.4

The basic medium was RPMI 1640 containing sodium bicarbonate (2g/l) to which the following were added:

MYOCLONE FOETAL CALF SERUM (MY-FCS)	10%
MERCAPTOETHANOL	$5x10^{-5}$M
TYLOSINE	60µg/ml
GLUTAMINE	2mM

WHEN REQUIRED:

HYPOXANTHINE, AMINOPTERIN, THYMIDINE (HAT)	0.176mg/l
	13.6mg/l
	3.9mg/l

OR

HYPOXANTHINE, THYMIDINE (HT)	0.1mM
	16µM

FREEZING MIXTURE

MYOCLONE FOETAL CALF SERUM (MY-FCS)	50%
DIMETHYL SULPHOXIDE (DMSO)	20%
CLONING MEDIUM (NO HAT OR HT)	30%

2.3 METHODS

2.3.1 CULTURED SPINAL CORD CELLS

2.3.1.1 STERILE TECHNIQUE

Tissue culture materials were either obtained sterile, or sterilised in a bench-top autoclave at 121°C, 20psi for 20 min. Media and solutions were also either purchased sterile, or sterilised by autoclaving as above. Heat sensitive solutions were sterilised by passage through a 0.22μm filter. Culture media were usually supplemented with antibiotics to reduce further the risk of infection.

All "open" culture procedures were performed in a sterile air-flow hood (Microflow Pathfinder, M.D.H.).

The cultures were stored in ventilated dishes or bottles in a clean LEEC MKII Automatic CO_2 incubator at 37°C. The atmosphere supplied was 5% CO_2 in air.

Equipment which had come into contact with human material was post-sterilised by autoclaving or immersion in 1% sodium thiosulphate solution before disposal. Waste human material was soaked in 1% sodium thiosulphate before being sealed in plastic bags and deep frozen at -20°C for eventual transport to a recognised human waste disposal incinerator at the Royal United Hospital, Bath, Avon.

2.3.1.2 PREPARATION OF PLASTIC AND GLASSWARE FOR TISSUE CULTURE

Plastic ware was supplied specially processed

for tissue culture and required no further treatment before use unless they were treated with collagen (Section 2.3.1.3 and 2.3.1.4).

Glass coverslips were boiled in 0.1M sodium hydroxide and rinsed extensively in running tap water and distilled water. They were then dried at 60°C before enclosure in a glass bottle for autoclave sterilisation.

Glass PTFE coated multitest slides were individually washed in warm 3% Decon solution with gentle rubbing. They were then rinsed sequentially in running tap water, distilled water, and double-distilled water, followed by drying at 60°C. They were individually wrapped in aluminium foil and tape before sterilisation in the autoclave.

2.3.1.3 EXTRACTION OF RAT TAIL COLLAGEN

The skin was completely removed from a single rat tail and the tendons were stripped from the bones with strong forceps, rinsed in water and transferred to 1:1000 glacial acetic acid (100ml) in water. This was stirred for 24h at 4°C and the insoluble material was sedimented by centrifugation in a MSE 18 high speed centrifuge at 2,5000 r.p.m. (500xg) for 2h at room temperature.

A protein assay was performed, on a small aliquot of the supernatant, by the method of Lowry et al. (1951) and the remainder was stored at 4°C.

2.3.1.4 COATING AND PHOTOPOLYMERISATION OF COLLAGEN

Riboflavin sodium monophosphate solution (0.05% w/v) was prepared in double distilled water, filter sterilised, and stored in the dark at 4°C for up to 6 months.

This solution was added to collagen in the ratio 1ml to 4mg (approx. 4ml) and the mixture was quickly applied to culture surfaces at 10µl per cm^2 in low level illumination in the culture hood, and evenly spread with a sterilised L-shaped glass rod. The surfaces were exposed to bright fluorescent light for 30 min to induce polymerisation (Masurovsky & Peterson, 1973).

Collagen-coated culture-ware was stored in the incubator at 37°C for up to 1 week prior to use. Before a cell suspension was added, the collagen was rinsed slowly 3 times with sterile water to leach out the residual yellow-coloured riboflavin and to re-hydrate the collagen itself.

2.3.1.5 PRODUCTION OF A SINGLE CELL SUSPENSION FROM FOETAL RAT SPINAL CORD

This was performed according to the method already established in the department (Digby et al., 1985).

A 15 day pregnant rat was killed by stunning and cervical dislocation. The fur and skin were sterilised with 70% ethanol before transferal to the culture hood. The uterus was dissected out and the foetuses liberated into sterile phosphate buffered saline in a petri dish.

The spinal cords were dissected free from surrounding tissue under a binocular microscope and transferred to Puck's Ca^{++} and Mg^{++}-free balanced salt solution (3ml)(see p 106) for composition). They were then minced with iridectomy scissors and transferred to a screw-top tube (12ml). DNAase I (1mg/ml in Pucks solution; 250µl) was then added, followed by Trypsin III (5mg/ml in Pucks solution; 250µl).

After an incubation period of 30 min at 37°C, the spinal cord fragments were washed in serum supplemented media (SSM)(p 106) for composition) gently centrifuged at 1500rpm (200g) for 5 min in an MSE Centaur bench centrifuge. The supernatant was discarded and fresh SSM (3ml) was added. The fragments were dissociated by gentle passage up and down a plastic pasteur pipette (trituration). This was repeated two or three times until all fragments were dispersed. The total volume (ml) of medium used was equivalent to the number of spinal cords used.

A viable cell count was performed on a small aliquot of the cell suspension (50µl) mixed with an equal volume of 1.5% Trypan blue dye. The number of cells was 5±0.5 million per spinal cord. The number of spinal cords was usually between 10 and 15.

The cells were seeded onto culture surfaces in an appropriate volume of SSM at 100,000 per cm^2. Other seeding densities were also tested on bare glass culture surfaces (up to 200,000 per cm^2).

2.3.1.6 BASIC PROCEDURE FOR CULTURE OF FOETAL RAT SPINAL CORD CELLS

The cultures were kept in a LEEC incubator at 37°C in an atmosphere of 5% CO_2 in air. The cells initially attach to the culture surfaces in small aggregates. Over the first three days these aggregates become connected with numerous processes. The culture surface between the aggregates becomes covered with a monolayer of predominantly large, flat fibroblast-like cells. On day 3 the SSM was replaced by serum-free medium (SFM) (see p 106 for composition), which was replenished every 3 days. Under these conditions the cell aggregates remained viable (ie. phase-bright and attached to the culture surface with high levels of enzyme activities) for approximately 3 weeks (Digby et al., 1985).

2.3.1.7 PRODUCTION OF TISSUE EXTRACTS FOR ADDITION TO CULTURE MEDIA

Skeletal muscle was dissected free from the limbs of 8-14 week-old suction terminated human foetuses and 12-20 week prostaglandin induced aborted foetuses. Hearts and livers were removed from the larger foetuses. Adult human skeletal muscle was dissected free from fat and connective tissue.

All tissues were then treated in the same manner. They were washed twice with gentle squeezing in cold phosphate buffered saline and then homogenised at 10% (w/v) in fresh PBS using an Ultra-turrax homogeniser.

The homogenate was then centrifuged at 100,000g (35,000 rpm) for 60 min at 4°C in a Beckman L5-50b Ultracentrifuge. The supernatants were filter sterilised and then stored frozen in aliquots at -20°C. Small samples were retained for protein assay.

On defrosting the extracts, a brown precipitate often forms which can be removed by centrifugation at 4,000rpm (2,600g) before addition of the supernatants to media over the range 0-200µg of extract per ml.

2.3.1.8 MORPHOLOGICAL EXAMINATION AND PHOTOGRAPHIC RECORDING OF CULTURES

The cultures were routinely examined on a Carl Zeiss "Jena" inverted phase contrast binocular microscope under tungsten lamp illumination. Live or fixed cultures were frequently processed by an immunofluorescence method and these were examined on a Zeiss Standard 18 microscope fitted with an epi-fluorescence condenser equipped with HBO 50W super-pressure mercury lamp for UV excitation. Cultures grown on multitest slides or glass coverslips could be examined under both low (x100) and high (x400) power magnification, while cultures grown in plastic culture dishes could be examined under low power only, if the dishes were inverted.

The cultures could be photographed by attachment of an Olympus OM 2R camera back to the trinocular head. The camera was loaded with Kodak ASA 400 black and white film for prints or Fuji ASA 400 colour reversal film for slides. Under tungsten lamp illumination best results

were obtained with a 1/30s exposure (low power) or ½s exposure (high power). Under UV illumination an exposure of 10-15s was found suitable depending on the strength of the fluorescence observed.

Black and white films were processed using Kodak D76 (negatives) and D163 (prints)developers according to manufacturer's instructions. Colour reversal film was returned to the manufacturer for processing.

2.3.1.9 ASSAY OF TOTAL PROTEIN IN CULTURES

The homogenised culture samples for protein assay contained 0.5% Triton X-100 non-ionic detergent. It was therefore necessary to use a modified Lowry protein assay for detergent extracts (Markwell et al., 1978) in which SDS is included to prevent precipitates being formed in the reaction tubes and cuvettes.

Standard curves were constructed over the range 0-100µg protein by using bovine serum albumin (BSA) as the standard. A standard solution of BSA in "homogenisation buffer" (potassium hydrogen phosphate 0.05M. di-sodium hydrogen phosphate 0.05M, sodium chloride 0.2M, EDTA 1mM and 0.5% v/v Triton X-100) (2mg/ml; 10-50µl) were pipetted into individual plastic tubes (3ml) in duplicate. The volume was adjusted (to 50µl) with buffer where necessary. Two tubes contained buffer (50µl) only as blank controls.

Solution A contained 2% sodium carbonate, 0.4% sodium hydroxide, 0.16% potassium sodium tartrate and 1% sodium dodecyl sulphate (all w/v in distilled water).

Solution B was 4% copper sulphate heptahydrate (w/v) in distilled water. Just prior to the assay, these two solutions were mixed in the proportion 100:1 respectively, and the resulting solution (C) (1ml) was added to each of the test tubes containing protein, with gentle mixing. After an incubation period of 10 min at room temperature, Folin and Ciocalteau's phenol reagent (100µl), diluted 50% with distilled water, was added to each tube with further mixing. The blue colouration which developed was read after 45 min in a Cecil CE 212 spectrophotometer at a wavelength setting of 750nm.

The optical density readings were plotted against the protein content of the samples. The standard curves were repeated several times on different days to ensure their reliability.

In order to assess the total protein present in spinal cord cell cultures it was first necessary to harvest and homogenise the cells. The test cultures were usually grown in 4 x 15mm dia. multi-well culture dishes. The medium was removed and the cultures were washed carefully twice with phosphate buffered saline pH7.4. The drained cultures could then be stored at -20°C/-80°C for simultaneous future assay (eg. for time-course studies).

Preliminary experiments indicated that a suitable volume in which to pool and homogenise a single 4-well dish culture was 300µl. This was achieved with 2 x 150µl aliquots transferred sequentially around the 4 wells. The cells were scraped from the culture surfaces into

the buffer using a metal spatula wrapped with PTFE tape to prevent cell adhesion.

The cells in buffer were transferred to a ground glass mini-homogeniser (Jencons,Ltd.,Beds., UK.) and disrupted by hand. It was found that with all cultures that a 10-50µl sample of this homogenate, when substituted for the BSA solution in the standard curve procedure, would give an optical density which fell in the appropriate linear range. It was then a simple procedure to calculate the total protein content of the homogenate.

The protein content of the cultures is usually expressed as µg per 15mm dia. well. Volumes were suitably adjusted when 35mm dish cultures were used.

2.3.1.10 ASSAY OF CHOLINE ACETYLTRANSFERASE ACTIVITY IN THE CULTURES

Choline acetyltransferase (ChAT) activity was measured by a modified procedure based on the method described by Fonnum (1969).

The radioactively labelled Acetyl-Coenzyme A was diluted in homogenisation buffer to a concentration of 2mM and a specific activity of 10µCi/µ mole. Aliquots (100µl) of this solution were stored at -20°C.

On the day of the assay, the following were added to one of the radioactively labelled Acetyl-CoA aliquots to give the reaction mixture: BSA (50mg/ml; 100µl), homogenisation buffer (600µl), 12.5mM choline chloride (100µl) and 1.0mM neostigmine bromide (100µl). This was sufficient for up to 50 micro assays. The reaction

mixture was dispensed into Eppendorf tubes (20µl/tube). A single assay consisted of 4 tubes: duplicates contained either reaction mixture alone or reaction mixture plus 10M formic acid (20µl) as a reaction inhibitor. This was repeated according to the number of culture dishes which were to be assayed.

The assays were performed at the same time as the protein assays and hence aliquots of the same cell homogenates were added to the reaction tubes.

The reaction catalysed by ChAT is:

$$[^{3}H][^{14}C]\text{-Acetyl-CoA+choline} \underset{}{\overset{\text{ChAT}}{\rightleftharpoons}} [^{3}H][^{14}C]\text{-Acetylcholine+CoA}$$

Preliminary experiments ascertained that production of radiolabelled acetylcholine is linear if 10µl aliquots of the homogenates were used and the tubes incubated for 10 min at 37°C. (NOT SHOWN). At the end of this period, the duplicate tubes which had not previously received formic acid had 20µl added to terminate the reaction.

To each reaction tube was then added freshly prepared sodium tetraphenylboron solution (15mg/ml in 2-Heptanone; 300µl). The tubes were stoppered and then shaken vigorously to mix the water and organic phases. The freshly synthesised acetylcholine was selectively extracted by ion-pairing with the sodium tetraphenylboron. The tubes were then centrifuged in an MSE microcentaur microfuge on high speed for 5 min. Aliquots (150µl) of the organic phase were removed from each tube and transferred to scintillation vials containing Optiphase-safe scintillant (5ml). The samples were counted for 1-2 min in a Packard Tricarb liquid scintillation

spectrophotometer.

A value for the rate of labelled acetylcholine production is obtained by the following equation:

$$\frac{\text{SPECIFIC CPM (ie. CPM NO FORMIC-CPM WITH FORMIC x 2 x EXTRACTION FACTOR x 30}}{\text{COUNTING EFFICIENCY x 2.2 x } 10^6 \text{ x SPECIFIC ACTIVITY AcCoA (μCi/μmole) x 10 min}}$$

= [^{3}H] OR [^{14}C] Acetylcholine formed μmoles/min/culture dish

NOTES

(a) The specific cpm is multiplied by two as only half of the organic phase is counted.

(b) The extraction of labelled acetylcholine by sodium tetraphenylboron in heptanone was shown by Fonnum (1969) to be approximately 95-98%. As comparative, rather than strictly absolute values were required, the extraction factor was therefore assumed to be 100% (ie. 1 in the equation).

(c) As a 10μl aliquot was used, the values are multiplied by 30 to give the total activity in the 300μl of homogenate from one 4-well dish.

(d) 2.2 x 10^6 is the standard cpm per μCi.

(e) The specific activity of the diluted labelled AcetylCoA was 10μCi/μmole.

(f) The figure was divided by the number of minutes incubation (10) to arrive at a final result of labelled acetylcholine production in μmoles/min/culture well.

The result could also be related to the protein assay obtained for the same dish to give the specific activity in μmoles/min/mg protein.

2.3.1.11 ASSAY OF SPECIFIC NEUROFILAMENT PROTEIN LEVELS IN CULTURES

The method was based on that reported by Doherty et al. (1984).

4-well culture dishes (frozen or fresh) were twice rinsed carefully with phosphate buffered saline and then fixed with 5% glacial acetic acid (0.5ml) in 70% ethanol at -20°C for 15 min. They were then rinsed with a buffered salt solution containing phenol red as pH indicator, until neutrality was re-established.

An initial incubation was performed with 5% animal serum (FCS, DHS, NRS or NGS were tested) in phosphate buffered saline. This solution (1ml) was added to each well and left at room temperature for 60 min. This blocks non-specific protein binding sites on the cultures and dishes, and also Fc receptor binding sites.

After this incubation, the blocking solution was replaced in two of the wells with the monclonal anti-neurofilament antibody supernatant (RPMI 1640 medium + 10% FCS + secreted monoclonal antibody). The other two wells in each dish received media which had not been exposed to antibody secreting hybridoma cells ("normal medium") to act as non-specific second antibody binding controls. The incubation period was 3h at room temperature.

The cultures were next washed three times with normal medium over a time period of 20-30 min. The second antibody (goat anti-mouse IgG-peroxidase conjugate) diluted 1:500 in normal medium was then dispensed at 0.5ml per well. It was left at room temperature for 2/3 h.

Following a final extensive wash period with phosphate buffered saline, the wells were filled with freshly made substrate solution (0.4ml) containing tetramethylbenzidine (10mg/ml in dimethylsulphoxide, DMSO; 0.1ml) and 30% hydrogen peroxide solution (1.5µl) in 0.1M sodium acetate buffer pH6.0 (10ml).

After 15-30 min at room temperature, the now blue-coloured substrate/product solution (0.1ml) was transferred to a 96 well microtitre plate containing 2M sulphuric acid (25µl) per well. This both stops the reaction and converts the blue product to a yellow colour. The optical densities were read at 450nm on a Dynatech minireader II. The results are proportional to the amount of neurofilament protein in the cultures.

2.3.1.12 PROCEDURE FOR THE DEMONSTRATION OF ACETYLCHOLINESTERASE ACTIVITY IN THE CULTURES

This was based on the "direct-colouring" thiocholine method of Karnovsky and Roots (1964) for teased or sectioned tissue.

Acetylthiocholine iodide (5mg) was dissolved in 0.1M sodium hydrogen maleate buffer pH6.0 (6.5ml). To this was added in sequence with mixing: 0.1M sodium

citrate (0.5ml), 30mM copper sulphate (1ml), distilled water (1ml) and 5mM potassium ferricyanide (1ml). The acetylcholinesterase inhibitor 1mM neostigmine bromide (1ml) was replaced for the water in the incubation medium as the control.

Cultures were washed and either used live or fixed with 5% acetic acid in 70% ethanol, or 4% paraformaldehyde in PBS. A suitable volume of test or control incubation medium was then applied and left for several hours at 37°C before examination on the Zeiss microscope.

2.3.1.13 IDENTIFICATION OF NEURONS IN THE CULTURES THROUGH TETANUS TOXIN BINDING

Live cultures grown on coverslips or 10-well multitest slides were incubated first with 5% SSM. This, and all subsequent incubations were for 1h at room temperature. The blocking solution was replaced with tetanus toxin diluted (to 10µg/ml) in 5% SSM. After careful washing in 5% SSM, the cultures were incubated with rabbit tetanus toxin antisera (1:100 in 5% SSM). Further washing was followed by incubation with anti-rabbit Ig-FITC conjugate diluted 1:100 in 5% SSM. There was a final wash (x2/3) in PBS before the cultures were mounted in PBS/glycerol (1:3) and examined on the Zeiss microscope.

2.3.2 MONOCLONAL ANTIBODIES

2.3.2.1 IMMUNISATION PROCEDURES

Balb/c mice were immunised with three different neural tissue preparations.

(a) Foetal rat spinal cord cell cultures were set up in 35mm dia. dishes and grown in SSM/SFM until day 15-18 to provide a source rich in neurones. Ten of these mature cultures were washed carefully with PBS and the cells were harvested by scraping into PBS (final vol. 0.5ml). The cells were injected into the peritoneal cavity of a 4-5 week old male Balb/c mouse without further addition or processing. An identical booster was given 3-4 weeks later.

(b) Preparations rich in synaptic membrane fragments were prepared from both cultured rat spinal cord cells and adult rat spinal cord tissue by the method of Young and Snyder,(1973). The tissue (10% w/v) was homogenised in 5mM Tris buffer, pH7.6, containing 50μM calcium chloride and 0.32M sucrose. The homogenate was centrifuged for 10 min at 1,000 g (4,000rpm). The supernatant was retained and the "nuclear" pellet was re-suspended and re-centrifuged as above. The two supernatants were pooled and centrifuged at 17,000 g (14,000 rpm) for 20 min at 4°C in a MSE 18 high speed centrifuge. The crude mitochondrial pellet thus obtained was "shocked" three times with 5mM Tris buffer pH7.6 (20 volumes) at 4°C. The suspension was re-centrifuged at 9,000 g (9,500 rpm) for 20 min to pellet the cytoskeletal proteins. Finally, the supernatant

was centrifuged at 48,000 g (26,000 rpm) for 20 min at 4°C in a Beckman L5-50b ultracentrifuge to produce the synaptic membrane pellet (P3) which was re-suspended in a minimal volume of PBS, assayed for protein, and stored frozen at -20°C. The defrosted preparation (100µg) was emulsified in Freund's complete adjuvant (0.25ml) and injected intraperitoneally into a young male Balb/c mouse. A booster injection was given some 3/4 weeks later with the preparation this time emulsified in Freund's incomplete adjuvant.

(c) A commercial preparation of choline acetyltransferase extracted from bovine brain was dissolved in PBS at a concentration of 0.5mg/ml. This solution (0.25ml) was mixed and emulsified with the same volume of Freund's complete adjuvant. This was then injected into young male Balb/c mice. After 3/4 weeks the procedure was repeated, this time with Freund's incomplete adjuvant.

2.3.2.2 MYELOMA CELL LINE CULTURE

During the period of immunisation, the X-63 8Ag myeloma cell line was continuously grown and sub-cultured. Until one week prior to the fusion procedure, the medium used, "cloning medium" (see p107 for composition) contained 0.13mM 8-Azaguanine to prevent the cells from spontaneously reverting to an aminopterin-insensitive form. This was later omitted. The cells were also occasionally tested for their sensitivity to aminopterin.

Care was taken to ensure that the cells were

healthy and growing in "log-phase". Their density was kept at around 0.5×10^6 cells per ml by regular sub-culture (usually every 2/3 days).

2.3.2.3 PROVISION OF A MACROPHAGE FEEDER CELL LAYER

Balb/c mice were killed by cervical dislocation and their fur and skin was sterilised by immersion in 70% ethanol. The skin was dissected back to expose the peritoneum. Sterile PBS (5ml) was then injected into the peritoneal cavity of each mouse. The abdomen of each mouse was massaged to suspend the peritoneal macrophages into the PBS which was then withdrawn by pipette through a hole cut in the peritoneum. Samples heavily contaminated with blood were discarded.

The macrophage suspensions were irradiated for approximately 4 min (2,000 rads), and then pelleted by centrifugation in an MSE centaur at 1500 rpm (200 g), at room temperature. The supernatant was discarded and the cells were resuspended in a small volume of normal cloning medium. A cell count was performed and the macrophages were then diluted to a suitable volume and seeded out into plastic culture ware at approximately 20,000 per cm^2. The macrophages were prepared the day before a fusion experiment and seeded out into 5 x 96 well flat-bottomed dishes in cloning media (50µl/well) containing hypoxanthine, aminopterin and thymidine (HAT supplement) (see p 107 for concentrations).

2.3.2.4 FUSION PROCEDURE AND SUBSEQUENT GROWTH OF HYBRIDOMAS

The fusion procedure was usually performed 5 days after the second injection of antigen into the mouse. In most cases, the serum of the mouse was tested for a positive reaction to the antigen/s 2 days prior to the fusion procedure.

On the day of the fusion procedure, polyethylene glycol (PEG, Merck 4,000) (4.5g) was added to PBS, pH7.4 (5.5ml), in a McCartney bottle. This was autoclaved and agitated while still hot to fully dissolve and disperse the PEG. A test on the resulting solution (when at room temperature) shows a pH of around 6.8. This is close to the lower peak for maximum fusion efficiency (K.Thompson, personal communication) and was therefore used unadjusted. It was kept at 37°C until needed.

The immunised mouse was killed by cervical dislocation, and sterilised. The spleen was dissected out and placed in a 90mm Petri dish. The cells were washed out with PBS (10ml) injected repeatedly into the spleen sac with gentle squeezing. The cells were then transferred to a sterile tube (12ml) and futher dispersed by gentle trituration. The contents were allowed to stand for a few minutes to allow any remaining lumps to settle. The cell suspension was then transferred to a sterile glass centrifuge tube (50ml).

The myeloma cells were then harvested. At a density of approximately 0.5×10^6 per ml, 2 x 50ml "T" flasks containing 15ml cell suspension each were

required to furnish 15 million cells. These were added to the spleen cell suspension to give an approximate ratio of 1 myeloma cell to 6/7 spleen cells.

The mixed cell suspension was centrifuged at 1500 rpm (200 g) for 10 min in a bench centrifuge. The supernatant was discarded and the warm PEG/PBS solution (1ml) was added dropwise over 60 sec to the pellet with constant agitation to prevent "clumping". This was immediately followed by warm PBS (1ml) added in the same manner. Further warm PBS was slowly added to make the total volume up to 20ml. The cells were then once again centrifuged and resuspended in warm normal cloning medium (20ml). This was dispensed into 2 x 50ml "T" flasks and incubated at 37°C overnight to allow for the completion of the cell fusion process. The next morning, the cells were pooled, centrifuged and resuspended in cloning medium (100ml) containing HAT supplement. The cell suspension was dispensed into the five macrophage-conditioned 96 well plates at 200µl per well. The plates were incubated at 37°C (10% CO_2 in air) for 5 days. Any infected plates, after 1 or 2 days, were discarded.

The medium was replenished at day 5 by withdrawal of medium (150µl) and replacement with fresh cloning medium + HAT (175µl). Inspection of the plates at this time usually revealed considerable cell death with debris accumulating around the circumference of the wells and "clearing" apparent in the centre.

After a further five days in the incubator, the medium was similarly replenished, except that cloning medium + HT supplement was used. Inspection of the plates

at this time (10 days) revealed considerable "clearing" of the wells. Occasionally there was some contamination from large, flat, fibroblast-like cells presumably derived from the capsule of the spleen sac. In extreme cases these cells would overgrow the cultures, resulting in their having to be discarded.

At day 15 the plates were again fed with cloning medium + HT supplement and re-examined. At this time some wells already contained large, visually obvious colonies of hybridoma cells. It is possible that some wells may contain up to 30 colonies derived from individual fusion events (K.Thompson, personal communication). The cells multiplied rapidly, and it was necessary to commence the staggered assay procedures when the colonies reached 50% confluency. The medium now required changing at least every two days.

2.3.2.5 SCREENING ASSAYS FOR SPECIFIC ANTIBODY PRODUCTION BY HYBRIDOMAS

2.3.2.5(i) INDIRECT IMMUNOFLUORESCENCE ASSAY

The main objective with the mouse immunised with whole rat spinal cord cell cultures was to produce a monoclonal antibody which specifically recognised neurofilament protein. Preliminary tests with a donated anti-neurofilament antibody (RT97 John Wood, Beckenham) showed that the main cell clusters and processes (neurons) were strongly and specifically stained by the following procedure:-

Rat spinal cord cell cultures were set up on

13mm dia. coverslips or 10-well multitest slides and grown as previously described (Section 2.3.1.6) until day 15-18. The cultures were then carefully washed with PBS and fixed by immersion for 15 min in 5% glacial acetic acid in 70% ethanol. They were then rinsed until neutral by repeated immersion in a balanced salt solution containing phenol red as indicator. The cultures were next covered with a layer of 5% animal serum (usually NRS) in balanced salt solution (BSS) (13mm dia. coverslips-250µl; 8mm slide wells -50µl), and left for 1h at room temperature to block non-specific binding sites. This was then replaced with the monoclonal antibody suitably diluted in 5%NRS/BSS (or the test hybridoma supernatant undiluted). The cultures were allowed to stand for 1h at room temperature. Controls were incubated with 5%NRS/BSS alone. Excess antibody was washed away by dipping the cultures in a large volume of 5%NRS/BSS and the cultures were covered with the appropriate volume of second antibody conjugated to fluorescein isothiocyanate diluted 1:100 in 5%NRS/BSS. A mixture of anti-mouse IgG and IgM or an anti-mouse polyvalent Ig (IgG, IgM + IgA) was used unless otherwise stated. The incubation was carried out in the dark for 1h at room temperature. Finally the cells were washed by dipping several times in BSS or PBS and mounted using glycerol in PBS (3:1) before examination on the Zeiss fluorescence-equipped microscope. A positive result was judged and graded with reference to the control incubation which received only the second antibody conjugate.

2.3.2.5(ii) DOT-IMMUNOBINDING ASSAY

This was the screening method used in the fusions where the mice had been immunised with commercial bovine ChAT. It was based on that reported by Hawkes et al. (1982).

Nitrocellulose paper was marked out into 4mm squares and cut into strips. The strips were washed in distilled water and then dried at room temperature. A solution (1mg/ml) of the bovine ChAT antigen in PBS was spotted onto the centre of each square (1µl) and allowed to dry for 30 min at room temperature. Control squares were spotted with BSA (1mg/ml in PBS).

The strips were washed in Tris-buffered saline, TBS (50mM Tris-hydrochloride, 200mM sodium chloride pH7.4) and, while still wet, cut up into their component 4mm squares and placed in the wells of 96 well microtitre plates. The unoccupied protein binding sites were then blocked by addition of 1% casein and 5% animal serum in TBS to each well (250µl). This was left for 30 min at room temperature with gentle agitation. The blocking solution was then replaced by the test supernatant or diluted antisera (150µl) and gently agitated for 3h at room temperature or overnight at 4°C. Controls received fresh blocking solution only.

The wells were next aspirated and filled with blocking solution several times over a period of 30 min to thoroughly wash the papers. The second antibody was then applied (anti-mouse IgG/M - peroxidase conjugate diluted 1:500 in blocking solution; 150µl) and left

for 2 h at room temperature. This was then removed and the filters thoroughly washed, as before, with TBS.

The enzyme substrate solution was prepared fresh by the addition of 0.4% 3-amino-9-ethylcarbazole (AEC) in dimethyl formamide (0.5ml) and 30% hydrogen peroxide (10µl) to 50mM sodium acetate solution, pH5.0 (9.5ml). This solution (100µl) was added to each well and gently agitated.

A positive result was indicated by the development of an insoluble brown product spot in the exact location of the original antigen spot. This was usually clearly visible within 30 min of substrate application. Colour development was terminated by several washes in distilled water and the nitrocellulose piece dried between filter papers.

2.3.2.6 CLONING OF HYBRIDOMA CELL LINES

The hybridomas growing in the wells which had given positive results in the screening procedures were immediately transferred to larger wells (15/16mm dia.) which had been pre-conditioned with fresh macrophages. The medium volume was increased to approximately 1ml with normal cloning medium (ie. HT could now be omitted). Two cloning techniques were used:-

2.3.2.6(i) CLONING BY LIMITING DILUTION

The cells in a selected well were counted for viability and serially diluted in fresh, normal cloning medium until their theoretical concentration

was either 5, 10 or 50 cells per ml. Each one of these dilutions was distributed (200µl/well) over freshly macrophage-conditioned 96-well plates. Two plates were used for the 5 cell/ml dilution to give a theoretical 192 wells each containing a single hybridoma cell. Only one plate was used for each of the other two dilutions to give a theoretical 2 and 5 cell/well distribution. The plates were incubated under normal conditions.

2.3.2.6(ii) CLONING BY MICROMANIPULATION

A reverse stage, inverted phase contrast microscope (Microtec, Micro Instruments Ltd., Oxford, UK) was placed in an open culture hood. The specially designed and constructed micromanipulating device was fitted with a sterile glass pasteur pipette which had ben drawn out to a fine point in a bunsen flame. A length of plastic tube attached to the pasteur served as a flexible mouthpiece.

Some cells from a selected well were diluted with normal medium in a 50mm dia. Petri dish, such that only a few at a time were in the field of view when observed under the microscope (HP x 200).

A small reservoir was taken up into the pasteur pipette to negate the tendency for capillary attraction. The tip of the pasteur pipette was then carefully brought into close contact with an individual cell which was taken up by gentle suction and transferred to a well of a 96-well plate containing macrophages and conditioned normal medium (250µl). This procedure was repeated with each well of two plates.

For either cloning method, the plates were fed with fresh medium at 5 day intervals. By day 15 it was usually possible to see colonies of hybridoma cells growing in many wells. The supernatants of wells containing colonies were screened for specific antibody by the appropriate assay method. Positive colonies were transferred to bigger wells and the entire cloning procedure was repeated a further two times to ensure the establishment of pure clonal cell lines. These could then either be suspended in deep frozen storage, or further expanded to produce pure antibody in greater quantitity.

2.3.2.7 DIRECT DEMONSTRATION OF IMMUNOGLOBULIN PRODUCTION BY HYBRIDOMAS

Antibody-producing hybridoma cells were removed from culture, concentrated by gentle centrifugation and washed once or twice with PBS. They were then resuspended in a minimal volume to give a "milky" suspension. Small drops (5µl) of this suspension were spread over the 8mm dia. wells of 10-well multitest slides. They were dried at 60°C. The cells were then either fixed by immersion of the slides in acetone at -20°C for 10 min and air-dried, or processed unfixed. Anti-mouse Igs (polyvalent or class specific) conjugated to FITC (1:100; 50µl) were then applied to test wells. Control wells were spotted with non-secreting X-63 8Ag myeloma cells and/or received no antibody-FITC conjugate.

After 1-2 h at room temperature the antibody-conjugate was rinsed carefully away and the slides were

mounted in PBS/glycerol (1:3). They were examined and photographed (sometimes under dual illumination) on the Zeiss microscope.

2.3.2.8 FREEZING OF HYBRIDOMAS

If the cells to be frozen were a confluent monolayer in a 15/16mm dia. well, then fresh normal medium (1ml) was exchanged for the existing medium. The culture dishes were placed on ice for 15 min and then ice-cold "freezing mixture" (50% FCS, 30% RPMI 1640, 20% dimethyl sulphoxide; 1ml) was added. The cells were suspended in the medium by careful trituration, and transferred to a 2ml sterile screw-top freezing vial on ice. The sealed containers were placed in the "vapour phase" of a liquid nitrogen storage vessel in such a position that they were cooled at the rate of approximately 1°C per min. After several hours they were fully immersed in the liquid nitrogen. When required, the cells were rapidly defrosted by placing the vials in a 37°C water bath. They were washed with two changes of fresh normal medium, with intermediate gentle centrifugation, before being returned to normal culture conditions.

Larger numbers of cells (up to 15 million) could also be frozen in this manner if they were first concentrated by gentle centrifugation and re-suspended in 1ml fresh medium.

2.3.2.9 PRODUCTION OF ASCITES FLUID

This procedure was based on that of Brodeur

et al. (1984).

Male Balb/c mice approximately 6 weeks old were primed for ascites tumour formation by the injection of pristane (2,6,10,14 tetramethyl-pentadecane; 0.5ml). They were left for 14 days and then injected with 3 x 10^6 hybridoma cells (washed and re-suspended in PBS (0.5ml)). The mice were observed over the next few days, and usually showed abdominal extension by day 10. The ascitic fluid was drained over the next few days by peritoneal puncture (19g needle) and stored frozen at -20°C.

2.3.2.10 IMMUNOGLOBULIN CLASS AND SUB-CLASS DETERMINATION

These were determined by a dot-immunobinding assay as previously described in detail(Section 2.3.2.5(ii)). The nitrocellulose pieces were spotted with goat anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 or IgM (1mg/ml; 1µl). After blocking, each test culture supernatant was incubated against the complete series of spotted nitrocellulose pieces. Antibody "capture" was detected with peroxidase-labelled rabbit anti-mouse Igs and substrate as before. A brown spot on one of the series of nitrocellulose pieces indicated tne immunoglobulin class of the monoclonal antibody.

2.3.2.11 SDS-PAGE AND WESTERN BLOT ANALYSIS

The electrophoresis was performed essentially as described by Laemmli (1970). The gels used were

approximately 15cm² and 2mm thick of 7.5% acrylamide-biacrylamide. An LKB vertical gel tank was used with the running buffer of Tris-HCl (5mM), glycine (39mM) and SDS (0.1%), pH8.3.

Samples of adult rat spinal cord tissue, foetal rat spinal cord cultures, or P3 membrane fragments from spinal cord (5mg/ml) were prepared by homogenisation in"dissolving buffer" (Tris-HCl 0.1M, 1% SDS, 10% glycerol, 0.14M 2-mercaptoethanol; pH8.9). A small volume of bromophenol blue (0.5%) was added and the samples were heated at 100°C in a boiling water bath for 5 min. Aliquots (50-100µl) of these samples were loaded into individual gel lanes. A mixture of standard proteins (Sigma High molecular weight markers; 20µl) was also loaded into one or two lanes.

Electric current was initially applied at 20mA (60V) to allow the samples to enter the gel (30 min). This was then increased to 30mA (200V). After 30 min a small volume of methyl green (0.25%) was added to each lane as a marker for subsequent transfer to nitrocellulose paper. This was repeated a few minutes before the termination of electrophoresis, which was usually complete in 5h (ie. when the dye front had travelled approximately 11cm).

Gels for direct staining were immersed in Coomassie brilliant blue R (1g/l) in fixing solution (methanol: acetic acid:water, 5:1:5) for a minimum of 1h and then destained in methanol:acetic acid:water (1:1.5:17.5).

Gels for electrophoretic protein transferal

were processed essentially as described by Towbin et al. (1979). A Biorad Transblot vertical tank was used, filled with buffer (25mM Tris, 192mM glycine, 20% methanol). The transfer was performed overnight at 20V (0.1 A).

Following electrophoretic transfer of the separated polypeptides to the nitrocellulose paper, individual lanes were cut out to produce many test strips. General protein staining was performed using Indian Ink in 0.01% Tween-20/PBS (1µl/ml). Other test strips were first incubated with a blocking solution (eg. 1% casein, 5-10% serum, 0.1% Tween in PBS) and then with the test monoclonal antibody containing supernatants (3h at 20°C or overnight at 4°C). Antibody binding was detected using anti-mouse IgG/M conjugated to peroxidase and the substrate solution as detailed for the dot-immunobinding assay (Section 2.3.2.5(ii)).

2.3.2.12 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The binding of monoclonal antibodies to their antigens was also studied using ELISA techniques. These were either performed on live or fixed whole spinal cell cord cultures in situ, or, alternatively homogenates or extracts of cultures or spinal cord was used as a potential antigen source.

For antibodies recognising intracellular antigens in whole cultures, the method used was identical to that decribed previously specifically to give an indication of comparative neurofilament protein levels (Section 2.3.1.11(ix)).

The binding of antibodies to the cell surfaces

of live cultures was also investigated by this semi-quantitative method. In particular, cultures were directly compared for surface antibody binding when they were grown under different medium conditions. Cultures were set up and grown for the first three days in 20% SSM. At day 3 some dishes were transferred to SFM while others were supplied with a low concentration (2-5%) of serum-supplemented SFM. Dishes were saved every three days and either tested immediately for surface antibody binding, or lightly fixed in glutaraldehyde (0.1% in PBS) and stored at 4°C.

Antibody binding to cell culture, spinal cord homogenate or P3 "membrane" extract was determined by a more traditional ELISA method. The homogenate or extract was diluted (to 10µg/ml) in 0.05M carbonate buffer pH9.6 and was then dispensed into the wells of a 96-well flexible microtitre plate (100µl per well). Control wells received only buffer, or another protein solution (eg. BSA). The protein was left to bind to the wells for 3h at 37°C. Excess was removed, and the wells were completely filled with blocking solution (1% casein, 5% animal serum in PBS). This was left <u>in situ</u> for 1h at room temperature and was then replaced by the test monoclonal antibody supernatant or control normal cloning medium (or antiserum diluted in blocking solution) for a further 2/3 h. The wells were then washed several times with blocking solution before being filled with anti-mouse IgG/M-peroxidase conjugate (1:500 in blocking solution; 100µl). After 2h at room temperature (or overnight at

4°C), the second antibody was discarded and the wells were copiously washed with PBS. The reaction was developed with the TMB substrate solution (see p 120) for 15-30 min. Sulphuric acid (25µl) was added to each well to stop the reaction, and the O.D. was read at 450nm.

3. RESULTS

INTRODUCTION TO RESULTS SECTION 3.1 CULTURED SPINAL CORD CELLS

Several factors have been shown to be beneficial to some neuronal tissue culture systems, in addition to the essential nutritionally complete medium, serum supplements and hormones. Some of these factors were therefore tested for their effects in our system, with the emphasis on muscle-derived neuronotrophic factors. The studies were extended to concentrate on previously untested human skeletal muscle extracts - a potential source of motor-neuronotrophic factors, and of possible relevance in MND.

The cultures were also used in studies to further investigate the reported immune system involvement in MND. Serum immunoglobulin binding to the cultures was assessed by ELISA, and the actual target cells for the binding was studied by immunofluorescence. Reported serum cytotoxic activity was also examined.

Prior to the commencement of this project, successful human foetal spinal cord dissociated cultures had not been reported. These were established, and used in some comparative experiments with rat spinal cord cell cultures.

Two established characterisation experiments were tested, and were successful in confirming the neuronal identity of the clusters and processes (tetanus toxin binding) and their considerable cholinergic nature (acetylcholinesterase activity).

3.1 CULTURED SPINAL CORD CELLS

3.1.1 COMPARISON OF CULTURES GROWN IN THE PRESENCE OR ABSENCE OF COLLAGEN

Foetal rat spinal cord cell cultures grown by the basic procedure (Section 2.3.1.6) on collagen coated glass and plastic ware (Section 2.3.1.4)developed as previously described by Digby et al. (1985). Small clumps of cells grew into large neuronal clusters with thick interconnecting processes, over a monolayer of glial and fibroblast-like cells. Protein and ChAT levels increased steadily until day 15-21 (see Fig.1 p140). The neuronal clusters remained viable (ie. phase-bright and attached to the substratum) for approximately 3 weeks.

Collagen-coating of the culture ware was, however, recognised to have several disadvantages as listed in the Discussion (Section 4.1.1). In view of these factors, some cultures, derived from single preparations, were grown in the presence or absence of collagen but otherwise under identical conditions. The cultures developed and appeared totally identical when monitored visually throughout their life span. In addition, a time-course study of comparative protein levels and choline acetyltransferase activity did not show any major differences between coated and uncoated dishes (see Fig.1 p140).

The small increase in protein levels (average 47µg per 35mm dia. dish) in collagen-coated dishes over

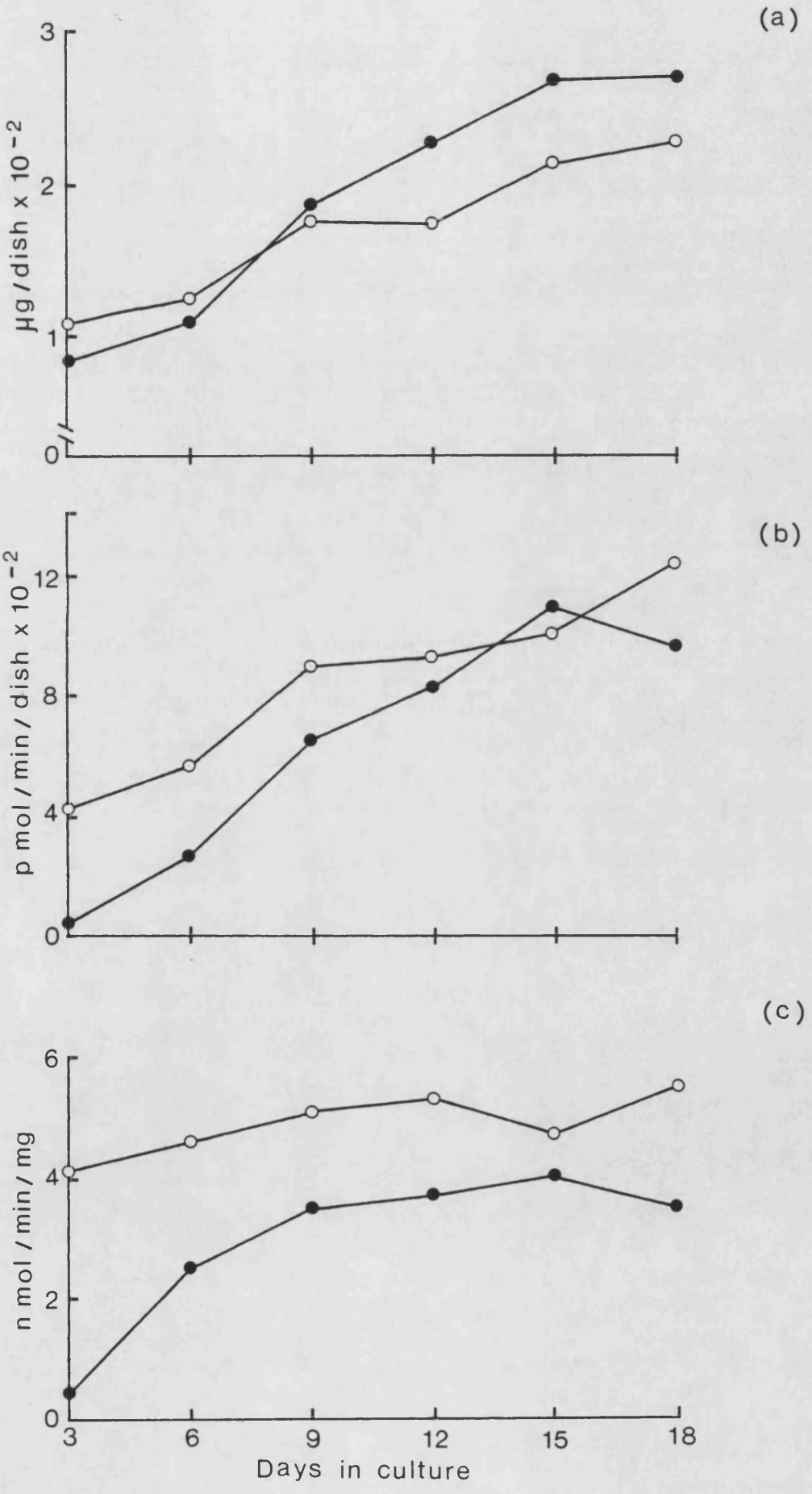
Fig.1 Time course study of the effect of collagen on the parameters of rat spinal cord cell cultures

The cultures were grown in 35mm dia. dishes which were either pre-coated with collagen (●) or not treated (o). All dishes were seeded from the same cell suspension at 100,000 per cm^2. Each point is the result from cell homogenate of three pooled culture dishes.

(a) Protein content per dish (μg)

(b) Choline acetyltransferase activity
([^{14}C]-Acetylcholine produced pmoles/min/dish)

(c) Specific choline acetyltransferase activity
([^{14}C]-Acetylcholine produced nmoles/min/mg protein)


(a)
3
2
1
0
µg / dish x 10⁻²
(b)
12
8
4
0
p mol / min / dish x 10⁻²
(c)
6
4
2
0
n mol / min / mg
3
6
9
12
15
18
Days in culture

days 12-18 was coincident with the amount of collagen used (50µg) to coat each dish. Collagen is detected in the protein assay used and the results suggest that it is more easily detached by the harvesting procedure in older cultures. This confirms the visual observations of collagen (plus culture) detachment frequently seen in older cultures.

In view of these findings, collagen coating was omitted and all subsequent results are based on cultures grown on untreated culture ware. Cumulated time-course data are presented in Fig.5 (p154) and Fig.11 (p175).

3.1.2 GROWTH OF CULTURES ON MULTITEST SLIDES

The use of multitest slides was judged to be essential in order to screen large numbers of hybridoma supernatants by the indirect immunofluorescence technique. Preliminary experimentation resulted in the establishment of the following successful procedure.

The slides were first washed individually by hand in warm 3% Decon solution and then rinsed sequentially in running tap water, distilled water and double-distilled water. The slides were dried at 60°C and then wrapped tightly in aluminium foil and autoclave tape prior to sterilisation at 121°C, 20psi for 20 min. Five slides were then placed face up in each 120mm² Petri dish and the cell suspension in SSM (100µl) was dispensed to every well at 2×10^5 cells/cm². The Petri dish and slides were then transferred carefully to the incubator

and left undisturbed for 24h (at 37°C, 5% CO_2). When examined after this period, the cells could already be seen to have formed clusters with a few fine processes interconnecting them. They appeared to be quite well attached to the surface.

The slides were carefully flooded with SSM (30ml) which ensured that the cell clusters remained firmly attached to the glass surface. The slides were replaced in the incubator and left for a further 2-3 days before transfer to SFM with subsequent changes every 3 days. Under these conditions the cultures grew and appeared in every respect to be identical to cultures grown in plastic ware. They remained viable and firmly attached to the glass surface for around 3 weeks, although they were usually used in the immunofluorescence screening procedure around day 15.

The average foetal rat spinal cord preparation provided up to 500 individual cultures by this method.

3.1.3 GROWTH OF CULTURES WITH VARIOUS MEDIUM SUPPLEMENTS

A number of variables were investigated in terms of their effects on the culture of spinal cord cells.

3.1.3(i) INCREASED POTASSIUM CONCENTRATION

The basic culture medium (Section 2.2) contains potassium chloride (KCl) at a concentration of 5.4mM. KCl solution was prepared at 2g per ml in double distilled water, filter sterilised, and then added to the basic medium to increase the concentration to 10, 20 or 40mM.

No adjustment was made to the medium to compensate for increased osmolarity (Section 1.1.3).

Foetal rat spinal cord cell cultures were set up in 35mm dia. non collagen-coated dishes and grown either in normal, or raised KCl medium. Cultures grown in 10 or 20mM KCL showed no visible morphological differences from those grown in 5.4mM KCl, and the survival time was not affected.

A time-course study was performed for comparison of protein content and choline acetyltransferase activity in cultures grown either in 5.4mM or 20mM KCl. Cultures were washed and stored frozen (-20°C) every 3 days for 21 days and assays performed simultaneously (see Fig.2 p 145). No differences were seen in protein content between the cultures. However choline acetyltransferase activity was 41.5% higher (average) between 15 and 21 days for cultures grown in 20mM KCl.

At 40mM KCl, the neuronal clusters became rapidly phase-dark and detached from the culture surface within a few days.

3.1.3(ii) ADDITION OF MITOTIC INHIBITORS

Deoxyfluorouridine (DFU) was made up at 1.5mg/ml in H-Eagles medium with uridine (U) added at 3.5mg/ml. The solution was filter sterilised and stored in frozen aliquots until needed. In an effort to produce cultures which were even purer in neurones than those grown from day 3 in SFM alone, the inhibitors were added to SFM at a final concentration of DFU 15µg/ml and U 35µg/ml.

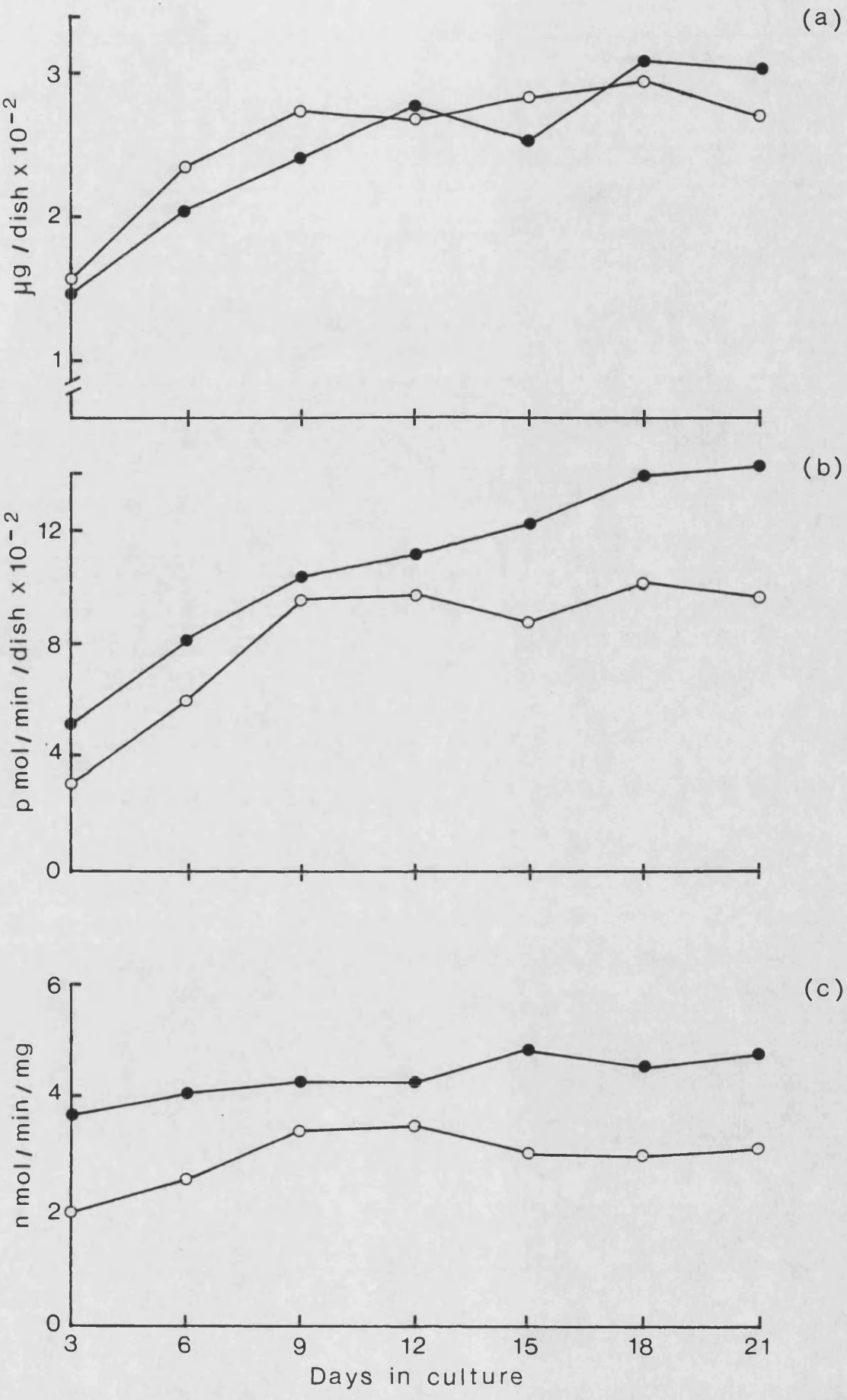
Fig.2 Time course study of the effect of elevated potassium chloride concentration on the parameters of rat spinal cord cell cultures

The cultures were grown in untreated 35mm dia. dishes in normal, 5.4mM (o) or elevated , 20mM (●) potassium chloride containing medium. All dishes were seeded from the same cell suspension at 100,000 per cm^2. Each point is the result from cell homogenate of three pooled culture dishes.

(a) Protein content per dish (µg)

(b) Choline acetyltransferase activity
([^{14}C]-Acetylcholine produced pmoles/min/dish)

(c) Specific choline acetyltransferase activity
([^{14}C]-Acetylcholine produced nmoles/min/mg protein)


(a)
μg / dish x 10⁻²
3
2
1
(b)
p mol / min / dish x 10⁻²
12
8
4
0
(c)
n mol / min / mg
6
4
2
0
3
6
9
12
15
18
21
Days in culture

This media was added to cultures at day 3. Thereafter, normal SFM was replaced as usual.

The cultures were monitored visually, and a time-course study performed with dishes saved at 3 day intervals for future assay of protein and choline acetyltransferase activity.

When uridine and deoxyfluorouridine were included in the SFM between days 3-6, the cultures were erratic and unreliable. They were very prone to detach from the culture surface, especially during medium changes and processing for immunofluorescent staining etc. In addition, they did not appear to survive in a healthy condition for as long as cultures grown in SFM alone.

Fig.3 (p 148) shows a comparison between cultures grown in the presence and absence of mitotic inhibitors. Protein levels and choline acetyltransferase activities are seen to be lower in the former case, early in the time-course. Levels appear to recover by day 15. However, the ease of detachment and early death of the cultures produces a sharp decline thereafter. Plate 4 shows that the presence of mitotic inhibitors, nevertheless, leads to very pure neuronal cultures.

3.1.3(iii) ADDITION OF LOW CONCENTRATIONS OF FOETAL CALF SERUM (FCS)

This was performed to determine whether long-term cultures could be maintained in a serum supplemented medium without the inclusion of mitotic inhibitors and without the excessive proliferation of non neuronal cells. It also served as a protein supplement control

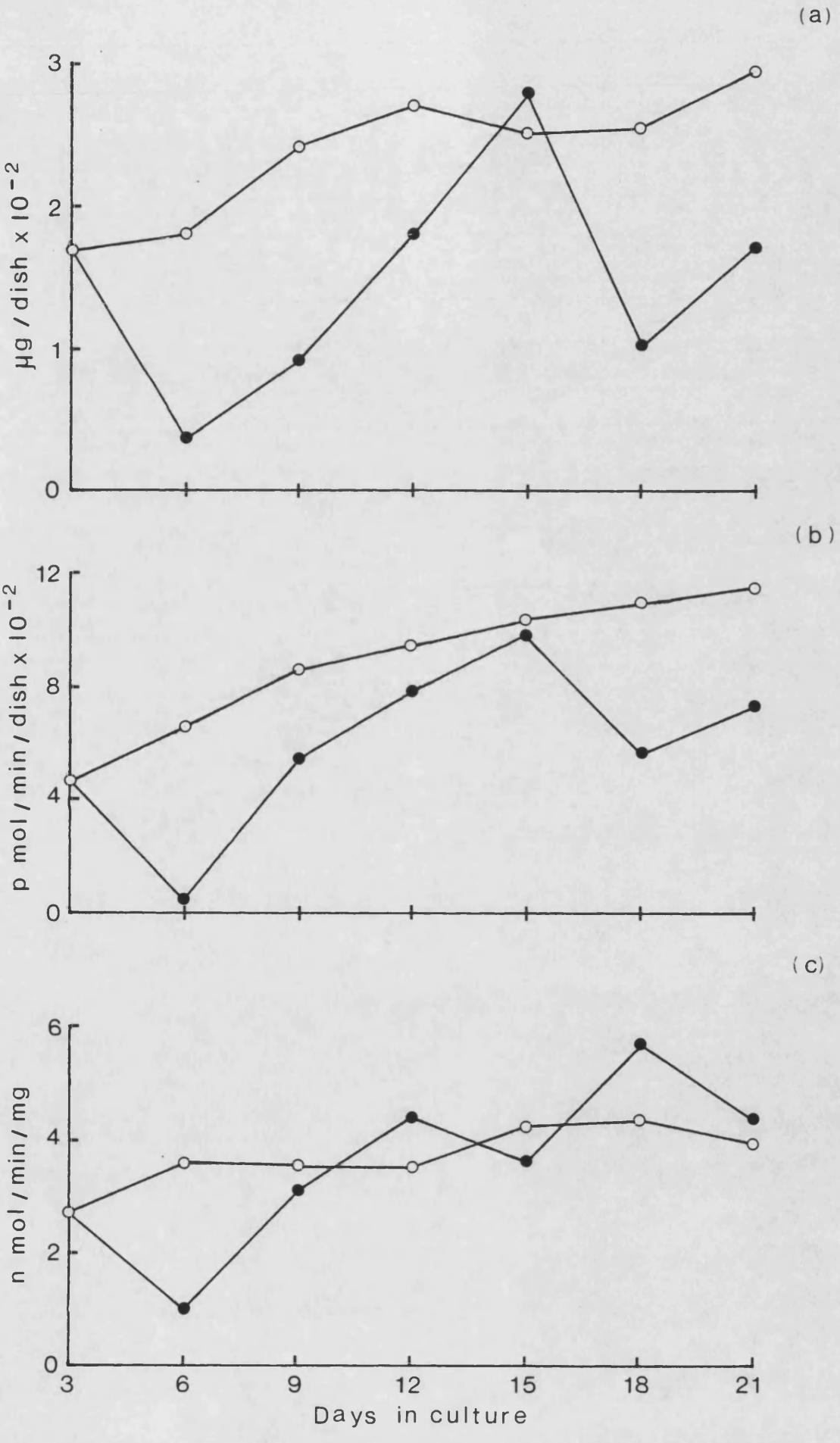
Fig.3 Time course study of the effect of mitotic inhibitors on the parameters of rat spinal cord cell cultures

The cultures were grown in untreated 35mm dia. dishes either in normal medium (o) or in medium supplemented with uridine, 35µg/ml, and deoxyfluorouridine 15µg/ml, between days 3 and 6 (●). All dishes were seeded from the same cell suspension at 100,000 per cm^2. Each point is the result from cell homogenate of three pooled culture dishes.

(a) Protein content per dish (µg)

(b) Choline acetyltransferase activity
([^{14}C]-Acetylcholine produced pmoles/min/dish)

(c) Specific choline acetyltransferase activity
([^{14}C]-Acetylcholine produced nmoles/min/mg protein)


(a)
3
2
1
0
µg / dish x 10⁻²
(b)
12
8
4
0
p mol / min / dish x 10⁻²
(c)
6
4
2
0
n mol / min / mg
3
6
9
12
15
18
21
Days in culture

for tissue extract experiments.

SFM was supplemented with FCS at 50µg/ml (0.125%), 100µg/ml (0.25%) and 200µg/ml (0.5%). These media were maintained from day 3 with visual observation until day 21 when all cultures were terminated and assayed for total protein, ChAT activity and neurofilament protein.

Other cultures were monitored visually with 1, 2 or 5% FCS retained in the SFM from day 3. The level of non-neuronal cell contamination of the cultures was observed to be directly related to the percentage of FCS added to the SFM from day 3.

The highest percentage which could be used was 5% and even then, by day 21, the neuronal clusters were practically obscured. 2% and 1% produced correspondingly improved cultures.

It was noticed that the serum did not appear to have a marked effect on the morphological appearance of the neuronal clusters and processes themselves - merely on the multiplication of the non-neuronal cells. The inclusion of serum did, however, appear to increase the survival time of the cultures from 21 days average to 30 plus.

A dose-response curve (Fig.8 p 164) was followed over the range 0-200µg/ml added FCS (ie. up to approximately 0.5% serum). It can be seen that there was only a very small increase in protein level (15%) at 200µg/ml. Neurofilament protein content was also only slightly increased. These two results were linked with a lack of significant morphological improvement. Choline

acetyltransferase activity was, however, increased by 36% at 200µg/ml.

3.1.3(iv) ADDITION OF THYROTROPIN RELEASING HORMONE (TRH)

TRH was made up at 10mM in distilled water, filter sterilised and stored frozen at -20°C. It was added to both SSM and SFM at 0.1mM and compared in culture with unsupplemented medium. Cultures were monitored visually and dishes saved every 3 days for protein and ChAT activity assays.

The inclusion of 0.1mM TRH in the medium (every 3 days from day 0) had no obvious morphological effect. Neither was there any discernible difference in the protein levels or choline acetyltransferase activity levels in the time course study. There was, however, a small consistent increase in specific ChAT activity (Fig.4 p 152).

3.1.4 GROWTH OF CULTURES IN HUMAN SKELETAL MUSCLE CELL CONDITIONED MEDIUM

Human myotube cultures were produced from fragments of human skeletal muscle (8-12 weeks), essentially as described in the basic procedure for foetal rat spinal cord cell cultures (Section 2.3.1.6) except that the trypsin/DNAse incubation was allowed to proceed for 45-60 min. The myoblasts produced were seeded at 200,000 per cm^2 in SSM (30ml) contained in 80cm^3 standard culture flasks.

Fig.4 Time course study of the effect of Thyrotropin releasing hormone (TRH) on the parameters of rat spinal cord cell cultures

The cultures were grown in untreated 35mm dia. dishes either in normal medium (o) or medium supplemented with 0.1mM TRH (●), added every 3 days in fresh medium from day 0. All dishes were seeded from the same cell suspension at 100,000 per cm^2. Each point is the result from cell homogenate of three pooled culture dishes.

(a) Protein content per dish (µg)

(b) Choline acetyltransferase activity
([^{14}C]-Acetylcholine produced pmoles/min/dish

(c) Specific choline acetyltransferase activity
([^{14}C]-Acetylcholine produced nmoles/min/mg protein

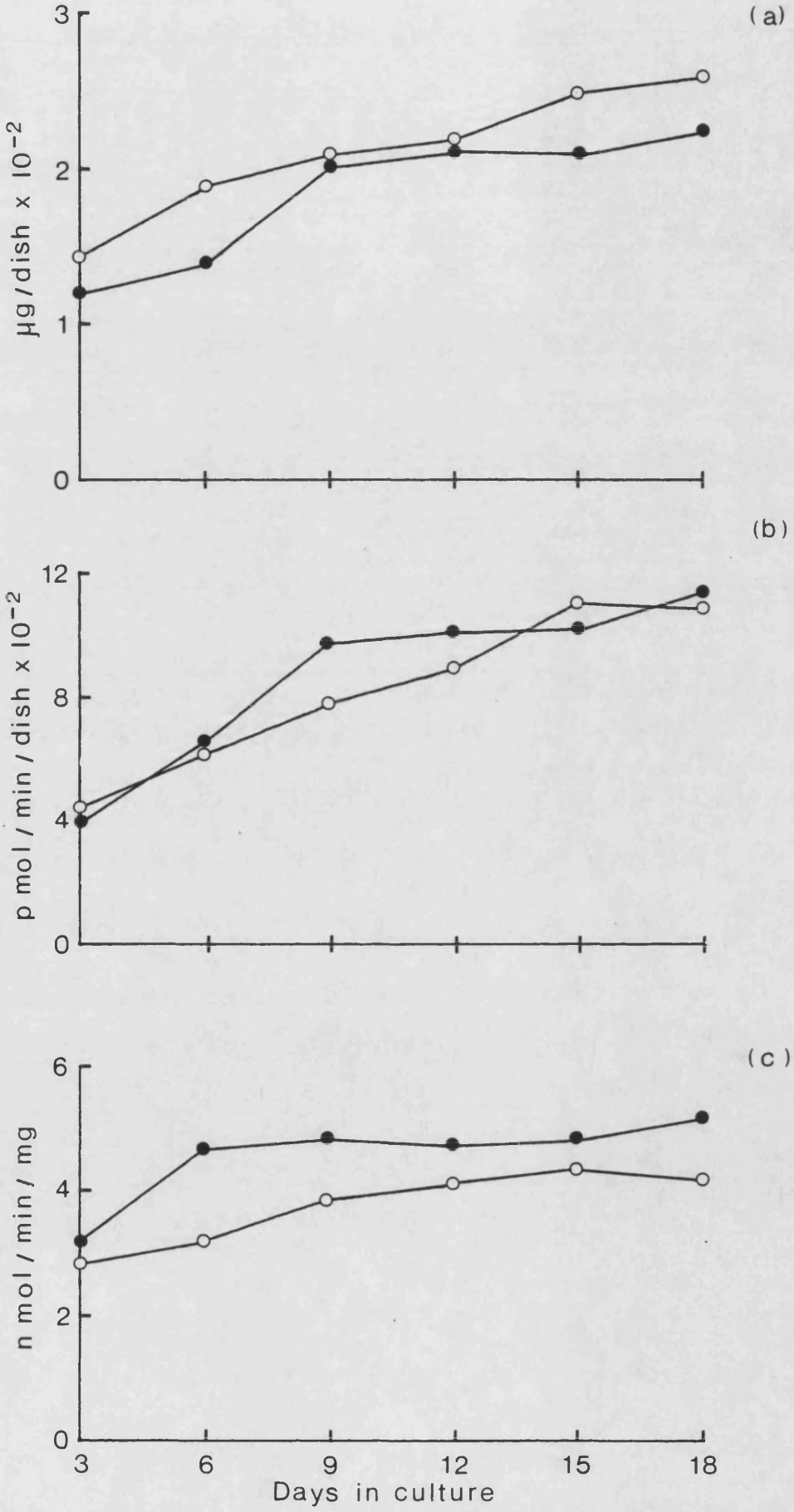

(a)
3
2
1
0
µg/dish x 10⁻²
(b)
12
8
4
0
p mol / min / dish x 10⁻²
(c)
6
4
2
0
n mol / min / mg
3
6
9
12
15
18
Days in culture

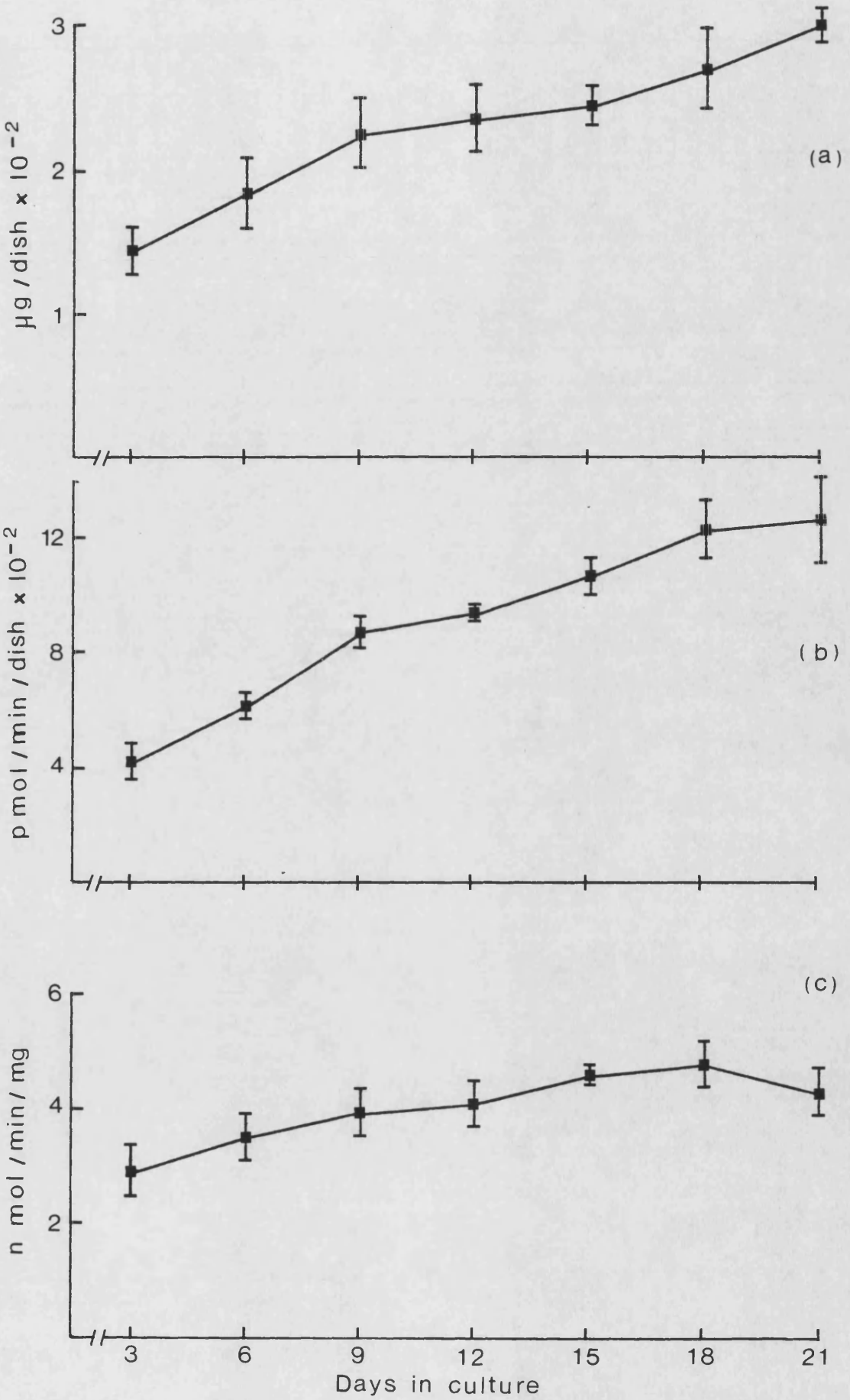
Fig.5 Cumulated time course parameters of rat spinal cord cell cultures grown in untreated 35mm dia. dishes

The results are accumulated from Figs. 1-4. Each point (days 3-18) is therefore the mean of four results (± S.E.M.). Each point day 21 is the mean of two results (± S.E.M.). All cultures were grown in 35mm dia. dishes which had not been collagen coated. The seeding density was 100,000 per cm^2.

(a) Protein content per dish (µg)

(b) Choline acetyltransferase activity
([^{14}C]-Acetylcholine produced pmoles/min/dish)

(c) Specific choline acetyltransferase activity
([^{14}C]-Acetylcholine produced nmoles/min/mg protein


3
2
1
µg / dish × 10⁻²
(a)
12
8
4
p mol / min / dish × 10⁻²
(b)
6
4
2
n mol / min / mg
(c)
3
6
9
12
15
18
21
Days in culture

The myoblasts multiplied for the first 3 days, completely covering the culture surface. At this time the SSM was decanted from the cells, centrifuged at 4,000rpm (2600g) for 10 min and the supernatant (conditioned SSM) stored at 4°C for up to 1 week, or frozen (-20°C) until required. The cultures were rinsed with SFM and then replenished with fresh SFM (30ml). The change to SFM caused the myoblasts to fuse rapidly into a network of myotubes. After a further 3 days the SFM was decanted from the cultures and treated as for conditioned SSM (see above). Fresh SFM was added for a further 3 days, by which time the myotubes had shrunk and withered away. The conditioned SFM was saved, and the cultures discarded.

Foetal rat spinal cord cells were seeded either into conditioned SSM or normal SSM. At day 3 the cultures were transferred to conditioned SFM or normal SFM respectively, with changes every 3 days as usual. The cultures were monitored visually and dishes saved every 3 days for future assays.

Cultures grown in conditioned medium throughout showed noticeable morphological improvements over days 15-24. The neuronal clusters appeared larger and the processes thicker and more numerous. The non-neuronal cells appeared unaffected.

The increase in culture size and process density was confirmed in a time course study which showed an average 40% increase in protein levels over days 15-24. This was paralleled by an average 48% increase in choline acetyltransferase activity over the same period. The

net result was that the specific activity of choline acetyltransferase remained fairly constant throughout the entire time course (Fig.6 p 158). This is clearly different to the factors previously tested which caused increases in choline acetyltransferase activity only, without parallel increases in protein levels, thus resulting in a consistent rise in specific activity.

3.1.5 ADDITION OF FOETAL HUMAN TISSUE EXTRACTS

All tissue extracts were prepared by the same method (Section 2.3.1.7). Cultures were set up from day 0 in SSM with or without the extract added. At day 3 the media was changed to SFM with or without the extract added, as appropriate. The cultures were visually monitored with fresh media added, and dishes saved for assay, every 3 days as usual. Alternatively, all assays were performed on a fixed day (usually day 21). Foetal calf muscle was also tested for comparison with human muscle.

Control experiments were performed to study the effects of dialysis on the extracts, or pre-incubation of the culture dishes with media containing extract.

There was an obvious morphological improvement in cultures grown in the presence of foetal human skeletal muscle extract (50µg/ml medium). As with conditioned medium, the neuronal clusters appeared larger and the processes thicker and more numerous. This was most noticeable over days 15-24.

The increase in cluster size and process density was confirmed in a time course study which showed an

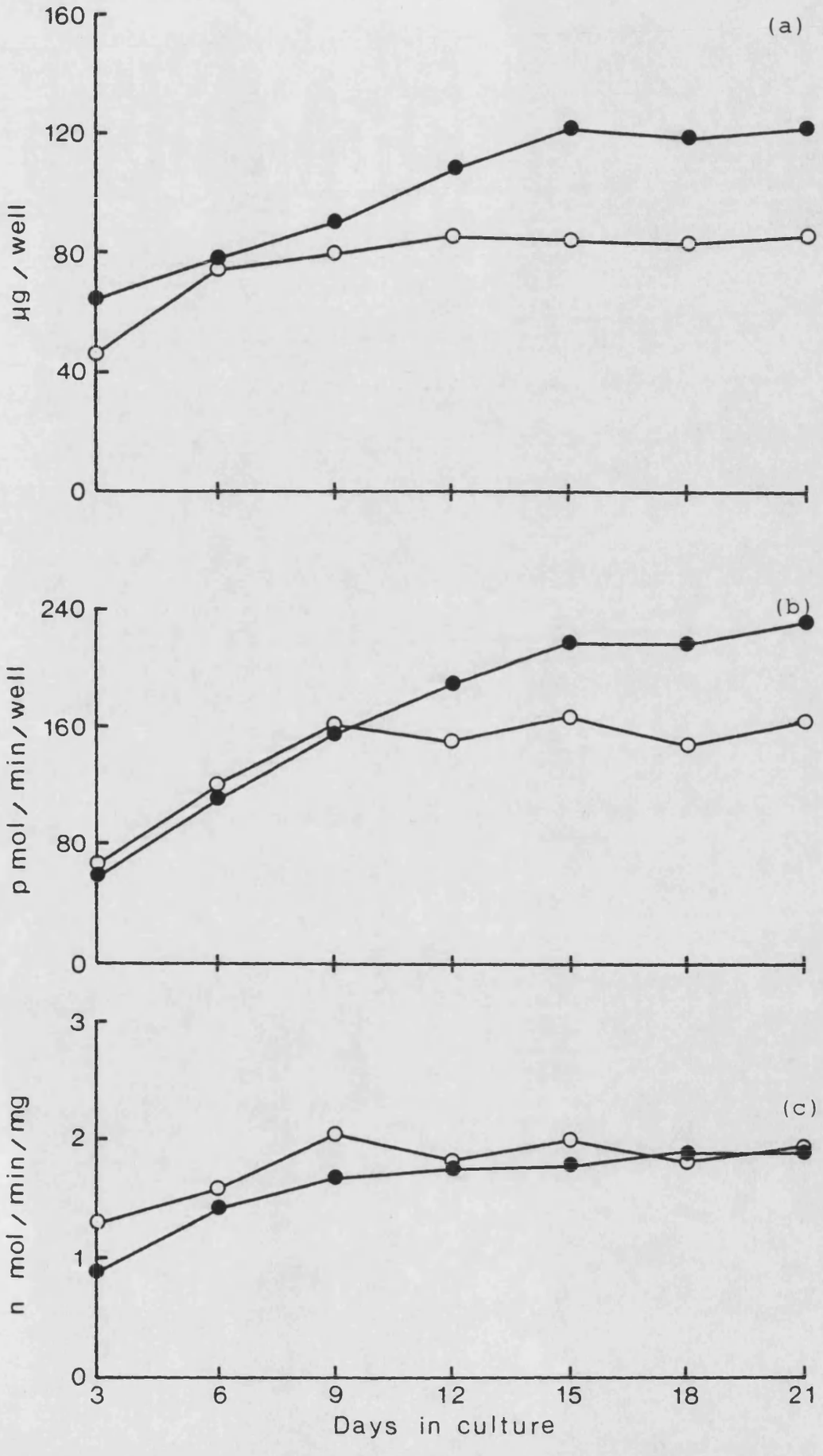
Fig.6 Time course study of the effect of human skeletal muscle cell conditioned medium on the parameters of rat spinal cord cell cultures

The cultures were grown in untreated 4x15mm dia. dishes in normal medium (o) or human skeletal muscle cell conditioned medium (●). All dishes were seeded from the same cell suspension at 150,000 per cm^2. Each point is the result from one dish ie. cell homogenate of four pooled culture wells (expressed per 15mm dia. well).

(a) Protein content per well (μg)

(b) Choline acetyltransferase activity
([^{3}H]-Acetylcholine produced pmoles/min/well)

(c) Specific choline acetyltransferase activity
([^{3}H]-Acetylcholine produced nmoles/min/mg protein


(a)
160
120
80
40
0
μg / well
(b)
240
160
80
0
p mol / min / well
(c)
3
2
1
0
n mol / min / mg
3
6
9
12
15
18
21
Days in culture

average 34% increase in protein levels over days 15-24. This was paralleled by an average 29% increase in choline acetyltransferase activity over the same period. The net result was that the specific activity of choline acetyltransferase was fairly constant over that period. (Fig.7 p 161).

The newly introduced assay for neurofilament protein also indicated an increase in levels (over days 18-24) for those cultures grown in the presence of the extract.

A dose-response curve was performed over the range 0-200µg extract per ml medium with all assays performed on day 21. The improvement in morphological appearance in the cultures grown in 200µg extract per ml medium is particularly striking by days 15-21 (See Plate 14). The assays confirmed this by demonstrating a linear increase in protein levels with an 118% increase at 200µg/ml. The choline acetyltransferase activity rose in parallel with the protein levels, but appeared to peak at 100µg/ml with an increase of near 70%. The net effect was a drop in specific activity at 200µg/ml (Fig.8 p 174).

The index of neurofilament protein levels showed a near linear increase over the range studied and appears to confirm the morphological observations of a large increase in the neuronal cluster and process density, while the non-neuronal cells are unaffected. This suggests a direct action of the extract on the neuronal structures.

Fig.7 Time course study of the effect of foetal human skeletal muscle extract on the parameters of rat spinal cord cell cultures

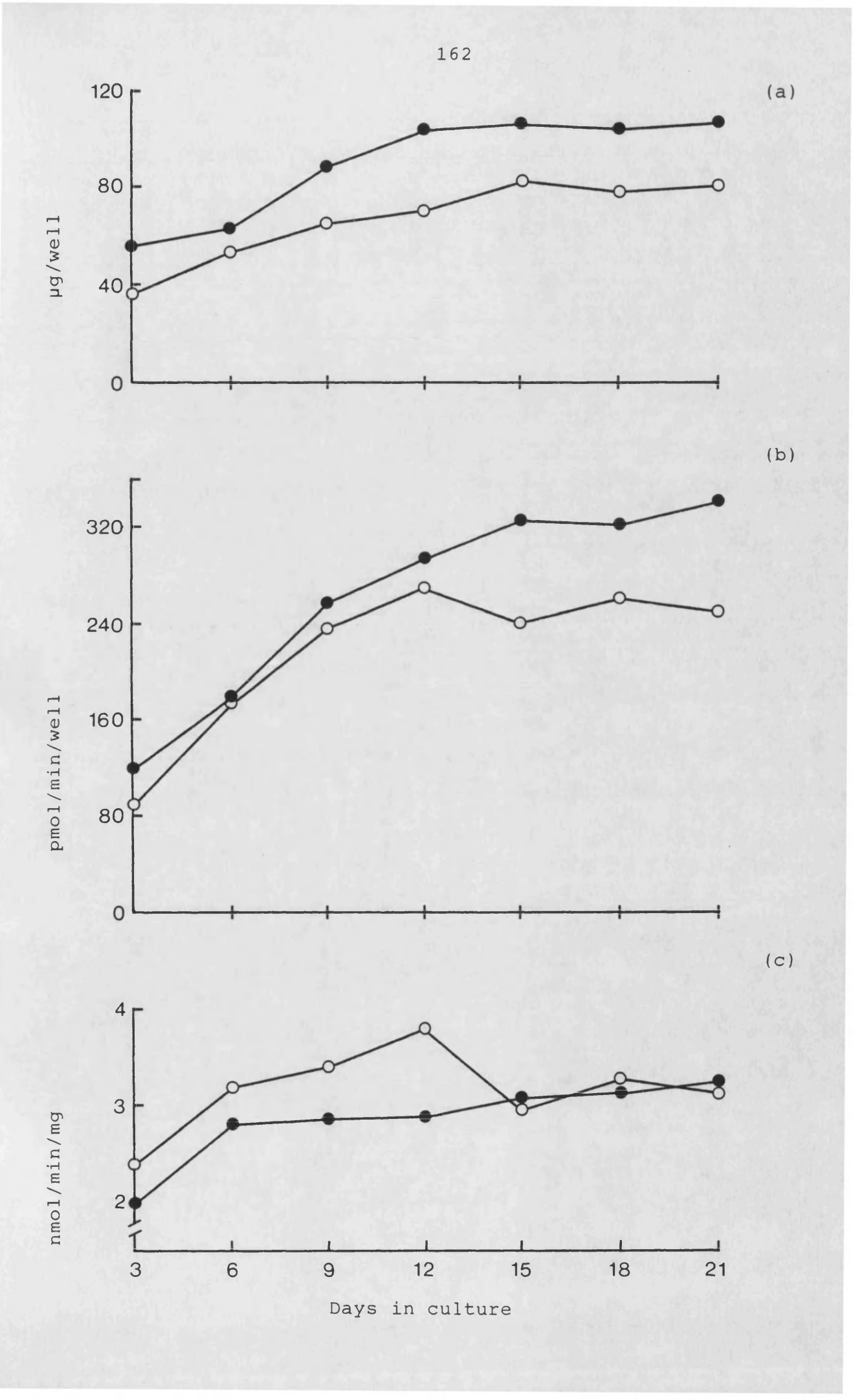
The cultures were grown in untreated 4x15mm dia. dishes in normal medium (o) or medium supplemented with foetal human skeletal muscle extract, 50µg protein per ml (●). All dishes were seeded from the same cell suspension at 150,000 per cm^2. Each point is the result from one dish ie. cell homogenate of four pooled culture wells (expressed per 15mm dia. well).

(a) Protein content per well (µg)

(b) Choline acetyltransferase activity
([^{3}H]-Acetylcholine produced pmoles|min|well)

(c) Specific choline acetyltransferase activity
([^{3}H]-Acetylcholine produced nmoles/min/mg protein)

(d) Neurofilament protein index (ΔOD.450nm)


(a)
120
80
40
0
µg/well
(b)
320
240
160
80
0
pmol/min/well
(c)
4
3
2
nmol/min/mg
3
6
9
12
15
18
21
Days in culture

(d)

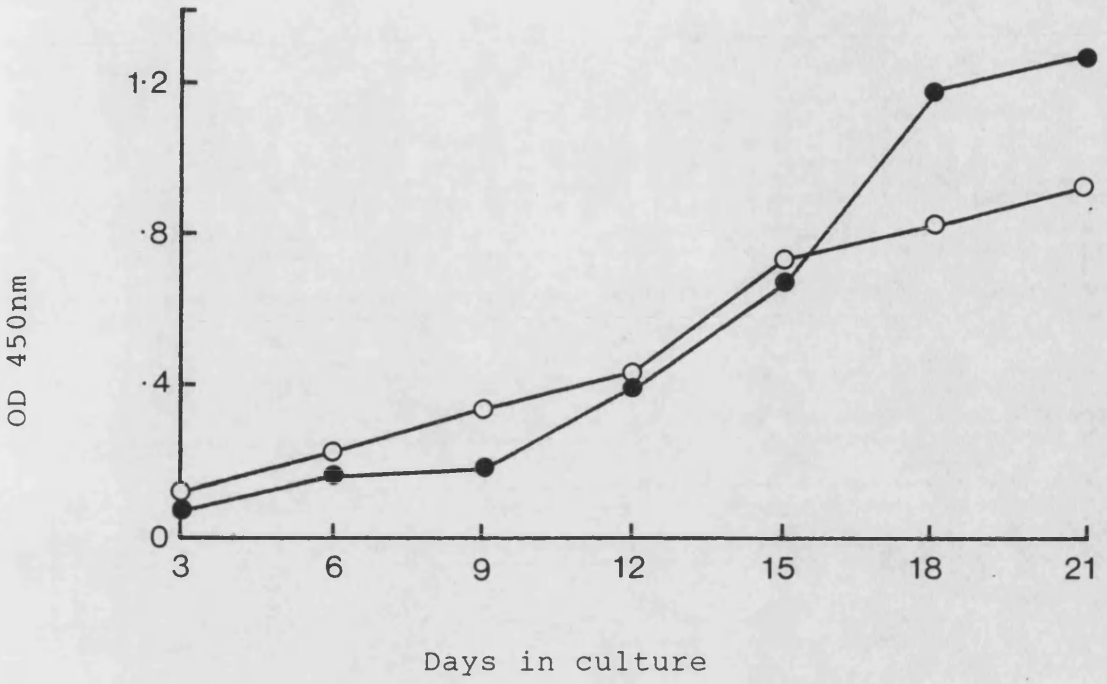


Fig.8 Dose response curves of the effect of foetal human skeletal muscle extract (and foetal calf serum) on the parameters of foetal rat spinal cord cells in culture

The extract (or FCS) was added to the cultures over the range 0-200µg protein per ml medium from day 0. The 4x15mm dia. dishes were all seeded from the same cell suspension at 150,000 cells per cm^2. The assays were all performed on day 21 and each point is the result from cell homogenate of four pooled wells (1 dish), expressed per well.

■ = Foetal human skeletal muscle extract

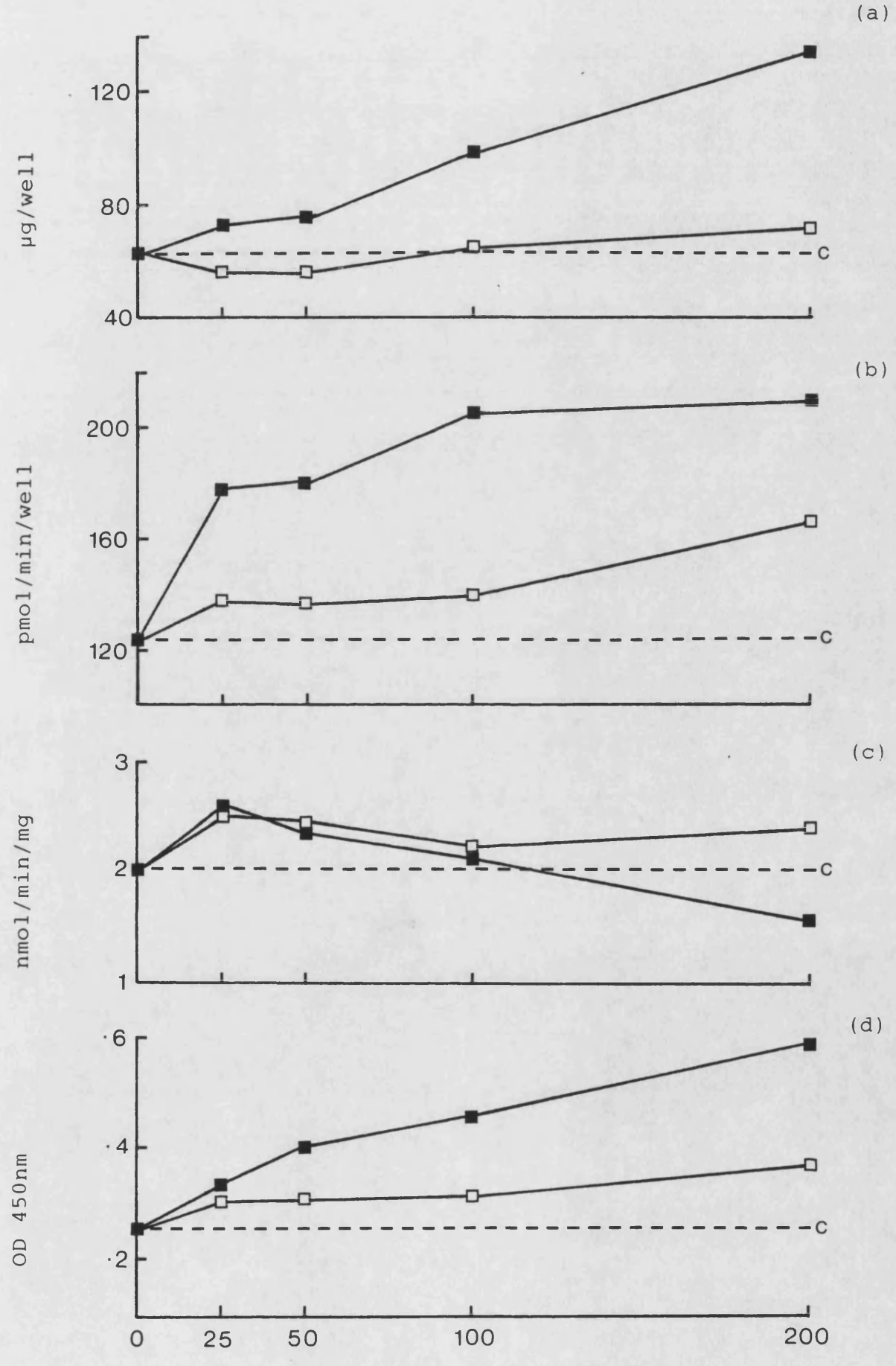
□ = Foetal calf serum (c = no addition)

(a) Protein content per well (µg)

(b) Choline acetyltransferase activity
([^{3}H]-Acetylcholine produced pmoles/min/well)

(c) Specific choline acetyltransferase activity
([^{3}H]-Acetylcholine produced nmoles/min/mg protein)

(d) Neurofilament protein index (ΔOD. 450nm)


(a)
µg/well
120
80
40
c
(b)
pmol/min/well
200
160
120
c
(c)
nmol/min/mg
3
2
1
c
(d)
OD 450nm
·6
·4
·2
c
0
25
50
100
200
µg protein/ml medium

An experiment was performed for comparison with these effects (see Table 5 p 167). It was found that:-

(a) Heating the extract to 100°C for 5 min destroyed the trophic activities (it became toxic in effect).

(b) Heat inactivation of the extract (56°C, 30') considerably reduced the "morphologic" trophic activity.

(c) Dialysis of the extract did not significantly reduce the trophic activities.

(d) Pre-incubation of the culture surface with the extract (200µg/ml SSM) did not itself have considerable trophic effects.

(e) Inclusion of human foetal heart extract (up to 200µg/ml) did not have comparable trophic activities.

(f) Inclusion of human foetal liver extract (200µg/ml) did,however,have moderate effects on the culture morphology, protein levels and choline acetyltransferase activity.

(g) The extract could not be used from day 0 as a substitute for serum.

It was with foetal human and foetal calf skeletal muscle extracts that an extension in the survival time of the cultures was first noticed. Up until this time, cultures had been studied over 21-24 days with termination at this point for assay. In cultures now maintained for longer periods it was seen that the neuronal clusters in serum-free medium alone were mostly phase-dark (dead)

Table 5 The extracts were all added to rat spinal cord cell cultures at 200µg/ml medium. Assays were performed on day 21. Each point is the result from four pooled 15mm dia. wells.

Extract tested	%increase in protein	%increase in ChAT activity	±change in specific ChAT activity
Foetal human skeletal muscle	118%	71%	-0.42
Foetal human skeletal muscle-dialysed	91%	46%	-0.54
Foetal human skeletal muscle-heat inactivated	15%	70%	+0.65
Foetal human skeletal muscle-pre-coating only	24%	6%	-0.30
Foetal human cardiac muscle	12%	10%	-0.03
Foetal human liver tissue	67%	34%	-0.46

and detaching from the culture surface by day 30. Cultures from the same preparation grown in SFM plus foetal human or foetal calf skeletal muscle extract (50-200µg/ml), however, appeared phase-bright, healthy and well attached to the culture surface. This was maintained until at least day 40.

Extension of culture survival was examined in more detail with extracts of adult human skeletal muscle (Section 3.1.6).

3.1.6 ADDITION OF ADULT HUMAN SKELETAL MUSCLE EXTRACTS

The extracts were prepared by the same method (Section 2.3.1.7) as for foetal tissue. The extracts were added to the cultures over the range 0-200µg extract per ml of media. Dishes were saved at 3 day intervals for time course studies, or alternatively all assays were performed on days 21, 30 or 40.

Time course studies (50µg/ml; Fig.9 p169) and 200µg/ml; Fig.10 p 172) and a dose-response curve (Fig.12 p 177 ; range 0-200µg/ml) using amputated human leg muscle as source, gave results similar to those obtained with foetal human skeletal muscle extract (see also Plates 14 and 15).

Five extracts from different amputated muscle samples were then tested (at 200µg/ml) with assays performed on days 21, 30 and 40 and compared to those cultures grown in SFM alone. The results are collected in Table 6 (p179).

It is clear from these results that the extract

Fig.9 Time course study of the effect of adult human skeletal muscle extract (50µg/ml) on the parameters of rat spinal cord cell cultures

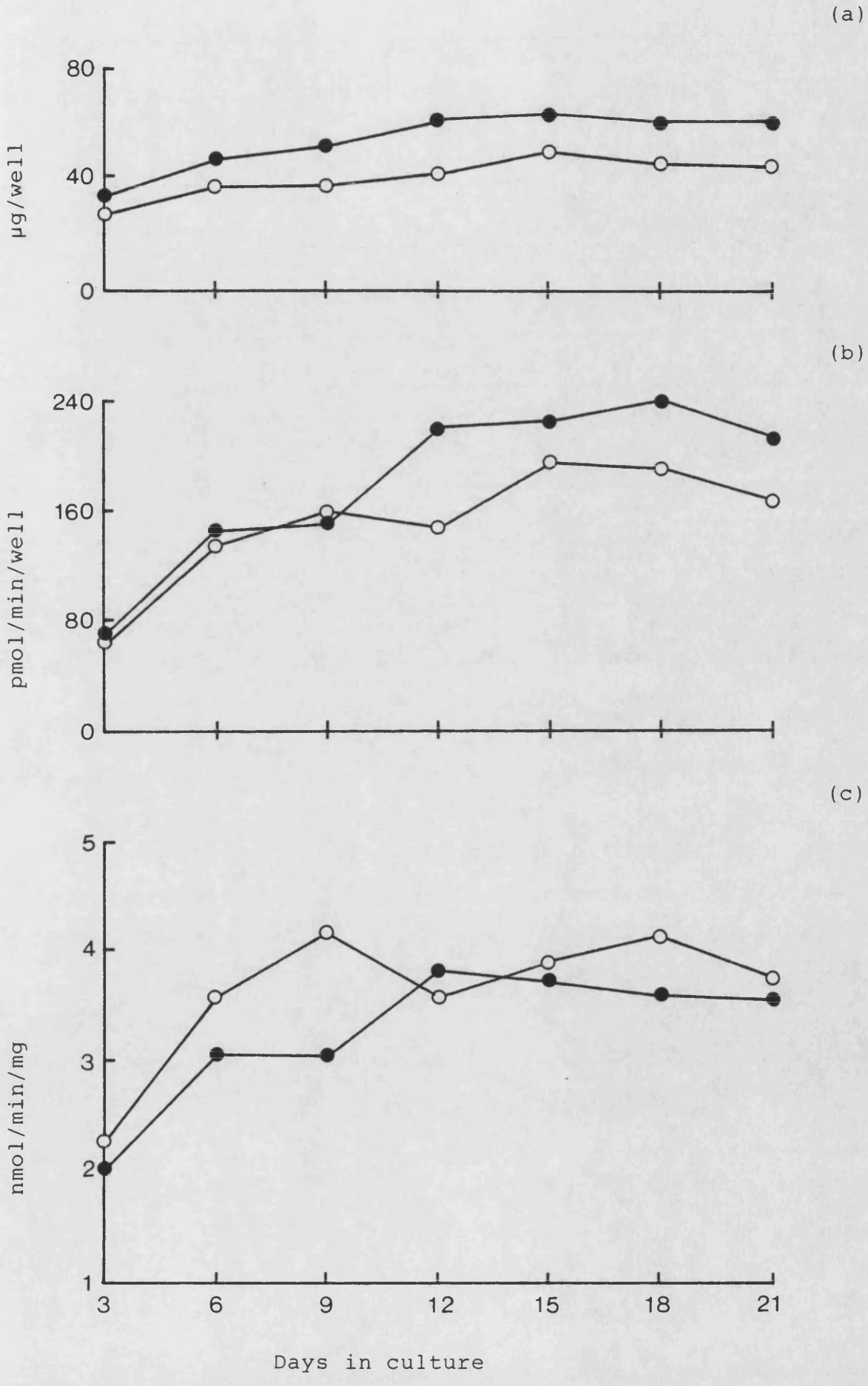
The cultures were grown in untreated 4x15mm dia. dishes in normal medium (o) or medium supplemented with adult human skeletal muscle extract from an amputated limb at 50µg protein per ml (●). All dishes were seeded from the same cell suspension at 150,000 per cm^2. Each point is the result from one dish ie. cell homogenate of four pooled culture wells (expressed per well).

(a) Protein content per well (µg)

(b) Choline acetyltransferase activity
([^{3}H]-Acetylcholine produced pmoles|min|well)

(c) Specific choline acetyltransferase activity
([^{3}H]-Acetylcholine produced nmoles/min/mg protein)

(d) Neurofilament protein index (ΔOD. 450nm)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80
40
0
µg/well
(b)
240
160
80
0
pmol/min/well
(c)
5
4
3
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1
nmol/min/mg
3
6
9
12
15
18
21
Days in culture

(d)

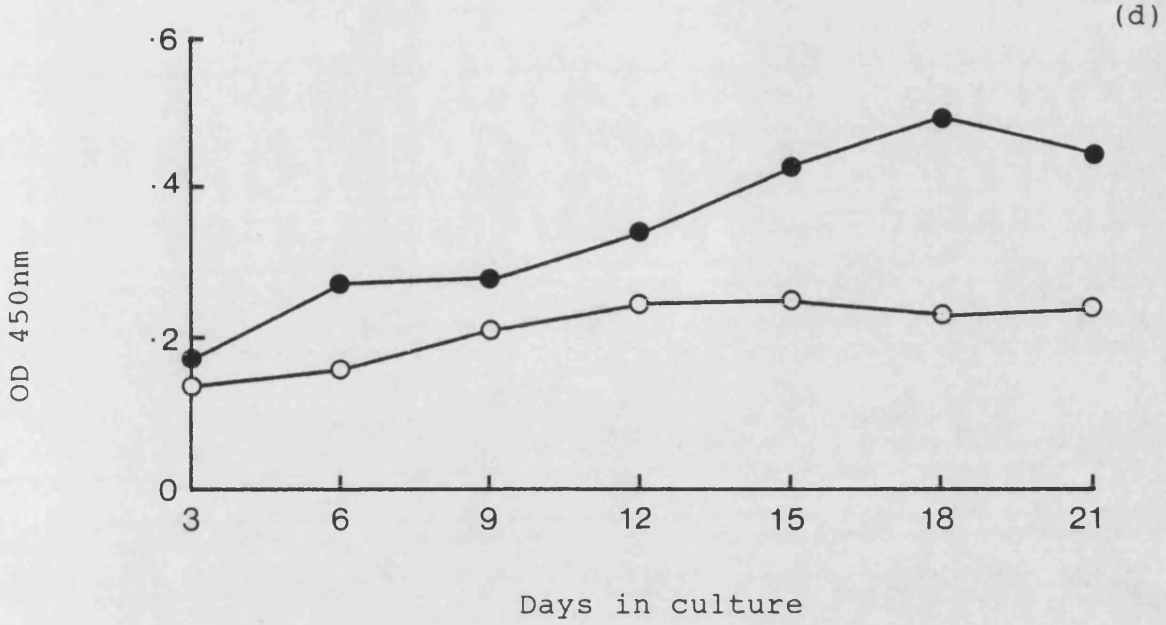


Fig.10 Time course study of the effect of adult human skeletal muscle extract (200µg/ml) on the parameters of rat spinal cord cell cultures

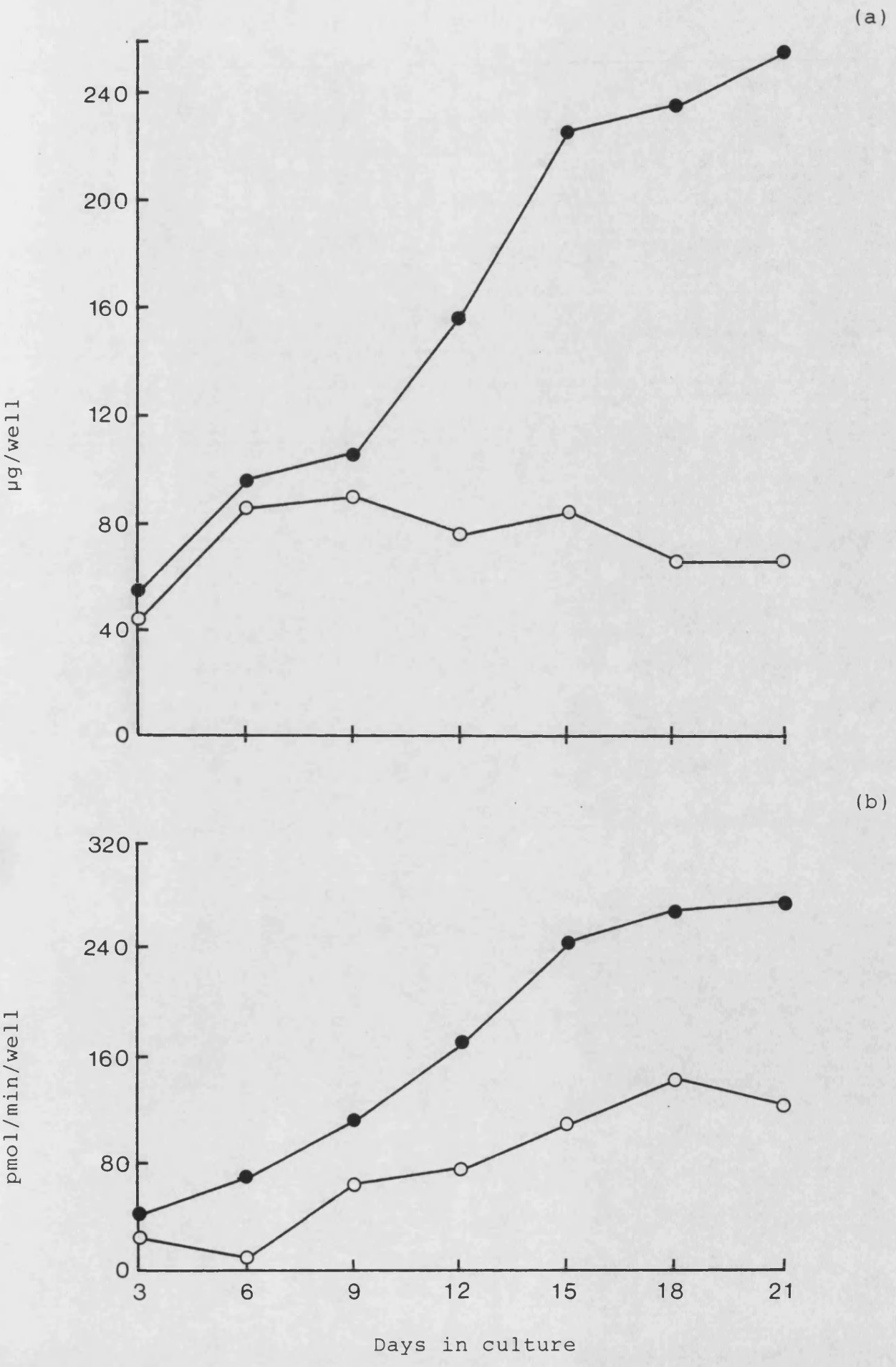
The cultures were grown in untreated 4x15mm dia. dishes in normal medium (o) or medium supplemented with adult human skeletal muscle extract from an amputated limb at 200µg protein per ml (●). All dishes were seeded from the same cell suspension at 150,000 per cm^2. Each point is the result from one dish ie. cell homogenate of four pooled culture wells (expressed per well).

(a) Protein content per well (µg)

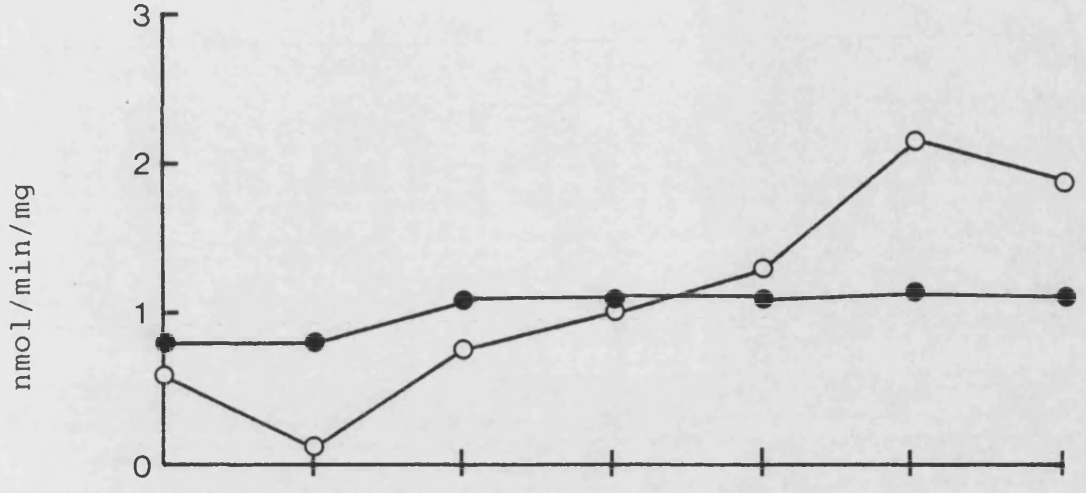
(b) Choline acetyltransferase activity
([^{3}H]-Acetylcholine produced pmoles|min|well)

(c) Specific choline acetyltransferase activity
([^{3}H]-Acetylcholine produced nmoles/min/mg protein)

(d) Neurofilament protein index (ΔOD. 450nm)


(a)
240
200
160
120
80
40
0
µg/well
(b)
320
240
160
80
0
pmol/min/well
3
6
9
12
15
18
21
Days in culture

(c)



(d)

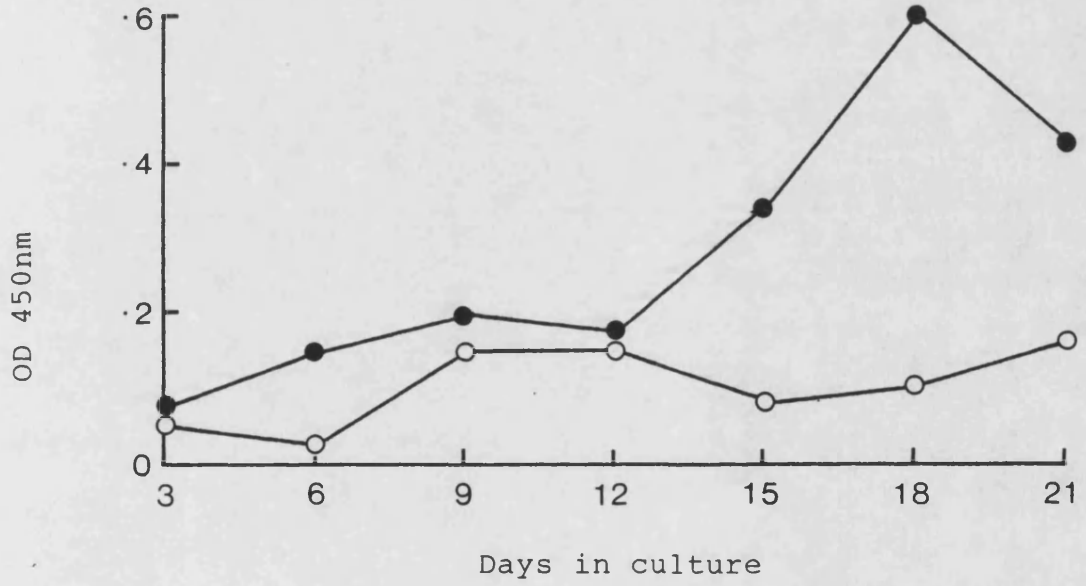


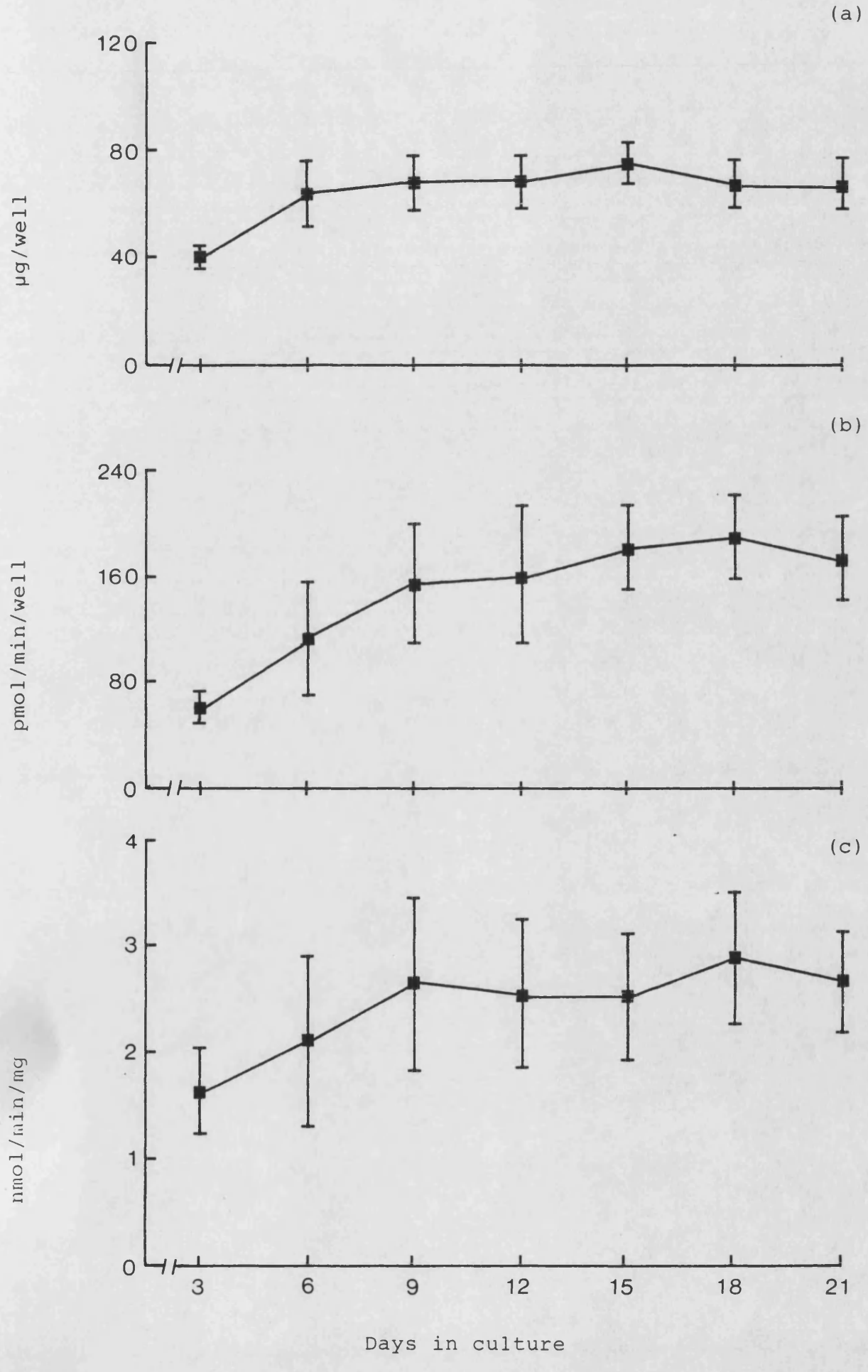
Fig.11 Cumulated time course parameters of rat spinal cord cell cultures grown in untreated 4x15mm dia. dishes

The results are cumulated from Figs. 6, 7, 9 and 10. Each point is therefore the mean of four results (± S.E.M). All cultures were grown in 4x15mm dia. dishes which had not been collagen coated. The seeding density was 150,000 per cm^2.

(a) Protein content per well (µg)

(b) Choline acetyltransferase activity
([^{3}H]-Acetylcholine produced pmoles|min|well)

(c) Specific choline acetyltransferase activity
([^{3}H]-Acetylcholine produced nmoles/min/mg protein)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120
80
40
0
µg/well
(b)
240
160
80
0
pmol/min/well
(c)
4
3
2
1
0
nmol/min/mg
3
6
9
12
15
18
21
Days in culture

Fig.12 Dose response curves of the effect of adult human skeletal muscle extract (from amputated limb) on the parameters of foetal rat spinal cord cells in culture

The extract was added to the cultures over the range 0-200µg protein per ml medium from day 0. The 4x15mm dia. dishes were all seeded from the same cell suspension at 150,000 cells per cm^2. The assays were all performed on day 21 and each point is the result from cell homogenate of four pooled wells (1 dish), expressed per well.

c = no addition

(a) Protein content per well (µg)

(b) Choline acetyltransferase activity
([^{3}H]-Acetylcholine produced pmoles|min|well)

(c) Specific choline acetyltransferase activity
([^{3}H]-Acetylcholine produced nmoles/min/mg protein

(d) Neurofilament protein index (ΔOD. 450nm)

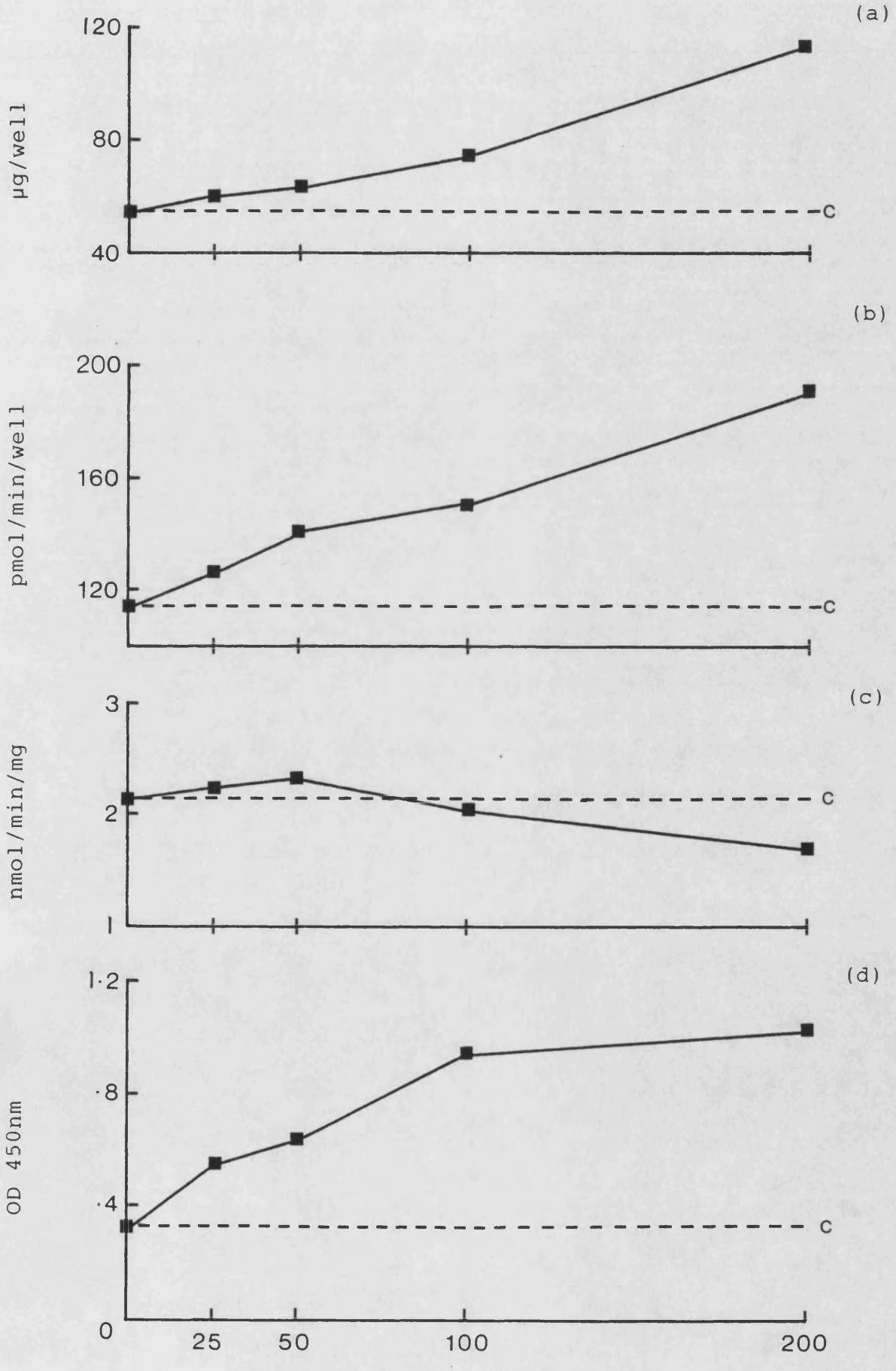

(a)
120
80
40
µg/well
C
(b)
200
160
120
pmol/min/well
C
(c)
3
2
1
nmol/min/mg
C
(d)
1·2
·8
·4
0
OD 450nm
C
25
50
100
200
µg protein/ml medium

Table 6 Effect of addition of human adult skeletal muscle extract (from amputated limb muscle) on the parameters of rat spinal cord cells in culture (extracts from 5 different patients were tested at 200µg/ml medium).

Assays	DAY 21			DAY 30			DAY 40		
	Control	+Extract	%increase	Control	+Extract	%increase	Control	+Extract	%increase
PROTEIN µg/well	83.4±2.1 n=5	217±12.9 n=5	160%	93 n=1	250±24.6 n=5	169%	dead & detached	341±16.5 n=5	-
ChAT activity [^{3}H]ACh. formed pm|min|well	155±7.6 n=5	275±12.9 n=5	77%	88 n=1	319±14.5 n=5	263%	dead & detached	330±11.1 n=5	-
Neurofilament protein index O.D. 450nm	0.24±0.04 n=5	0.51±0.05 n=5	113%	0.12 n=1	0.39±0.06 n=4	225%	dead & detached	0.42±0.05 n=4	-

promotes large inceases in all of the parameters and these are sustained until at least day 40. The neuronal clusters in the control cultures on the other hand are mostly dead and detached by day 30.

A dose-response curve, Fig.13 p 181 (range 0-200µg/ml) performed with extract from a sample of normal biopsied abdominal rectus muscle gave similar results to the other muscle extracts tested ie. striking morphological improvement, a sharp rise in choline acetyltransferase activity peaking at 100µg/ml (110% increase), and near linear rises in protein levels (100% at 200µg/ml) and neurofilament protein levels.

The three extracts from different normal small biopsy samples tested at 50µg/ml with assays at day 21 (through neccessity) showed consistent increases in the parameters. These increases were also reflected at day 40 in cultures which had received extract (at 50µg/ml) from seven different, larger normal biopsy samples (Table 7 p 183).

3.1.7 PREPARATION, GROWTH AND DEVELOPMENT OF FOETAL HUMAN CELL CULTURES

3.1.7(i) PRODUCTION OF SINGLE CELL SUSPENSIONS FROM FOETAL HUMAN SPINAL CORD

Because of the force produced by the suction termination equipment and the narrow bore of the tubing leading to the 'waste' container, only the very youngest foetuses (6-8 weeks) were found reasonably intact. Older foetuses (8-14 weeks) were considerably disrupted. In

Fig. 13 Dose response curves of the effect of adult human skeletal muscle extract (normal) on the parameters of foetal rat spinal cord cells in culture

The extract was added to the cultures over the range 0-200µg protein per ml medium from day 0. The 4x15mm dia. dishes were all seeded from the same cell suspension at 150,000 cells per cm^2. The assays were all performed on day 21 and each point is the result from cells pooled from 4 wells (1 dish), expressed per well.

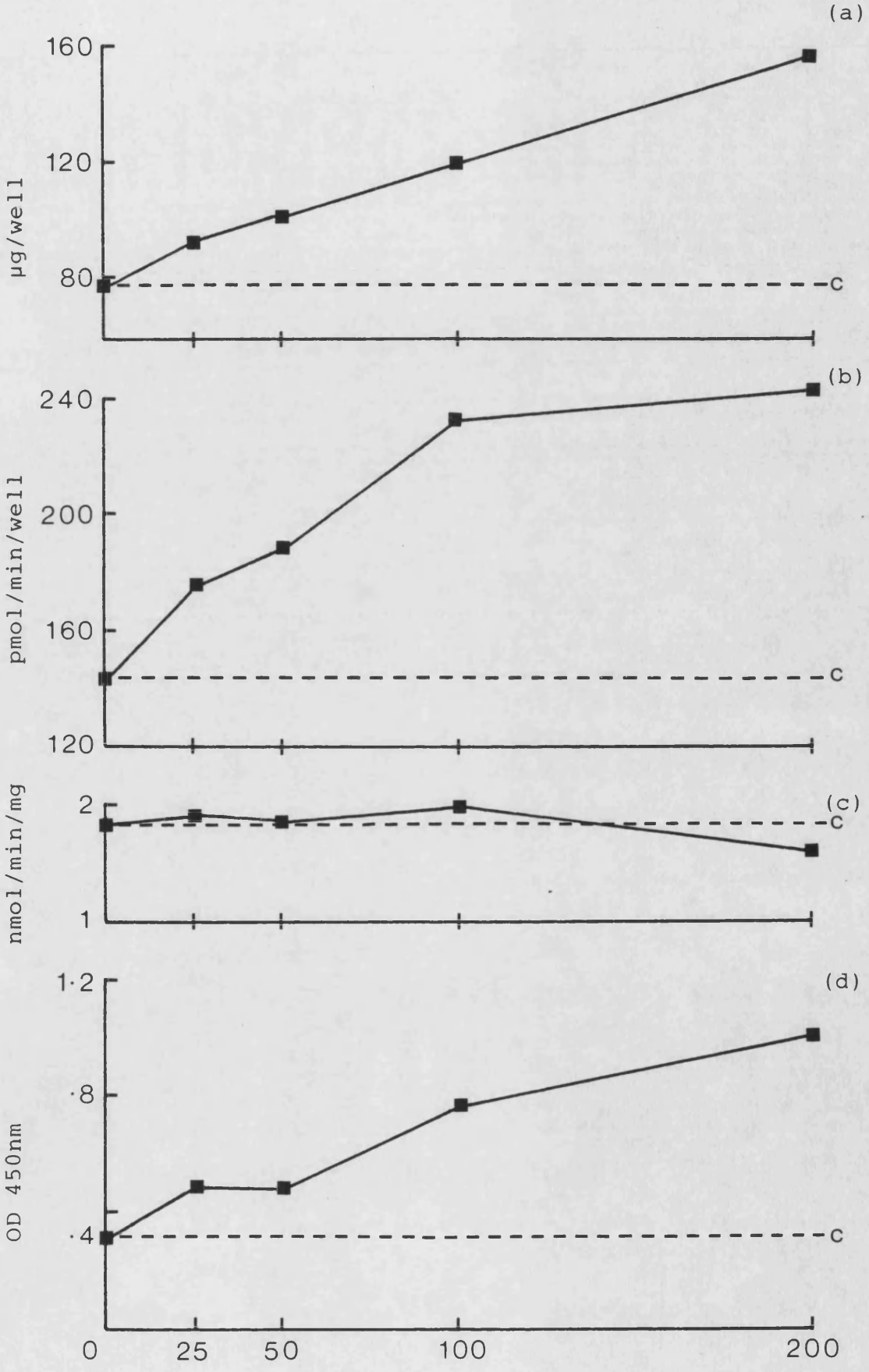
c = no addition

(a) Protein content per well (µg)

(b) Choline acetyltransferase activity
([^{3}H]-Acetylcholine produced pmoles|min|well)

(c) Specific choline acetyltransferase activity
([^{3}H]-Acetylcholine produced nmoles/min/mg protein)

(d) Neurofilament protein index (ΔOD. 450nm)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160
120
80
µg/well
C
(b)
240
200
160
120
pmol/min/well
C
(c)
2
1
nmol/min/mg
C
(d)
1·2
·8
·4
OD 450nm
C
0
25
50
100
200
µg protein/ml medium

Table 7 Effect of addition of adult humah skeletal muscle extract ("normal") on the parameters of rat spinal cord cells in culture (extracts from two groups; n=3,day21; n=7,day40, are shown-see text; tested at 50µg/ml medium).

Assays	DAY 21			DAY 40		
	Control	+Extract	%increase	Control	+Extract	%increase
PROTEIN µg/well	66±1.7 n=3	101±4.0 n=3	53%	dead & detached	93.9±3.6 n=7	-
ChAT activity [^{3}H]ACh.formed pm/min/well	149±4.1 n=3	216±4.8 n=3	45%	dead & detached	211±6.3 n=7	-
Neurofilament protein index O.D. 450nm	0.10±0.01 n=3	0.18±0.01 n=3	80%	dead & detached	0.15±0.01	-

these cases, sections of vertebrae were collected in the hope that they might contain some spinal cord tissue. The foetal parts were rapidly transported in H-Eagles medium containing Gentamycin (100μg/ml) and fungizone (2.5μg/ml).

Identified spinal cord tissue was processed in an identical manner to that for foetal rat spinal cord (Section 2.3.15). Appropriate adjustments were made to volumes of reagents etc. to allow for the difference in quantity of tissue obtained.

3.1.7(ii) MAINTENANCE OF DEVELOPING FOETAL HUMAN SPINAL CORD CELL CULTURES

The growth and development of dissociated cell suspensions, prepared from the spinal cords of 7-14 week human foetuses, were studied in a variety of different media under different conditions. The key observations which eventually dictated the successful protocol for maintenance in serum free medium are listed below:

(a) Cultures prepared from spinal cords of older, disrupted foetuses (9-14 weeks) never produced a neurone-rich culture, whatever the medium and conditions tested. The cultures produced were almost entirely composed of large, flat fibroblast-like cells. The cell count performed using trypan blue prior to seeding was between 5-8 x 10^6 viable cells per whole cord. There were, however, many dead cells present.

(b) Cultures prepared from the spinal cords of the smallest (2cm long), most intact foetuses (7-8 weeks), initially developed in a similar manner to that of rat cultures. Small clusters of cells were rapidly formed and a few fine processes could be seen to connect them.

(c) The development of the cultures was judged to be best when the normal SSM was additionally supplemented with the components of SFM. This medium, designated Human Medium-A, HM-A (see Section 2.2 for composition) was therefore routinely employed. Reasonable cultures were also seen in SFM supplemented with 10% human serum.

(d) The cultures did not survive if they were transferred to SFM before a confluent monolayer of non-neuronal cells had been formed. The multiplication of the non-neuronal cells was slower than in the rat cultures, and confluency was not achieved until day 7-9. Under these conditions the cultures would survive for around three weeks. Cultures grown throughout in SSM remain viable for longer, although the distinct neuronal clusters become largely obscured by non-neuronal cells after approximately 3 weeks in culture.

The human cultures never attained the degree of development seen in the rat cultures. The neuronal clusters were always smaller and more widely spaced, and the processes

'thinner' and less numerous.

The protocol finally developed is summarised below.

(1) Spinal cord tissue from 7-8 week human foetuses was dissected by the method described for rat spinal cord cells (Section 2.3.1.5).

(2) Cells were seeded at 2.5 x 10^5 cells per 15mm dia. well (150,000 per cm^2) in HM-A(1ml). (Collagen coating does not appear to improve the cultures).

(3) The cultures were incubated at 37°C in 10% CO_2/air and the medium was replaced with fresh, warm HM-A (1ml) on days 3 and 6. The HM-A could be replaced with SFM from day 9 and replenished every 3 days thereafter.

3.1.7(iii) CHARACTERISATION OF FOETAL HUMAN SPINAL CORD CELL CULTURES

Comparison of protein and choline acetyl-transferase activities of cultures from a single foetus transferred to SFM at day 9, or maintained in HM-A throughout are shown in Fig.14 p 187.

The cultures have been photographed at different developmental stages (see Plates 16, 18, 19), under varying media conditions (see Plate 16) and after histochemical and/or immunochemical staining procedures (for further details see sections 3.1.8, 3.1.9 and Plates 16, 17, 18 and 19).

In addition the cultures have been used to a limited extent in studies primarily for comparison with

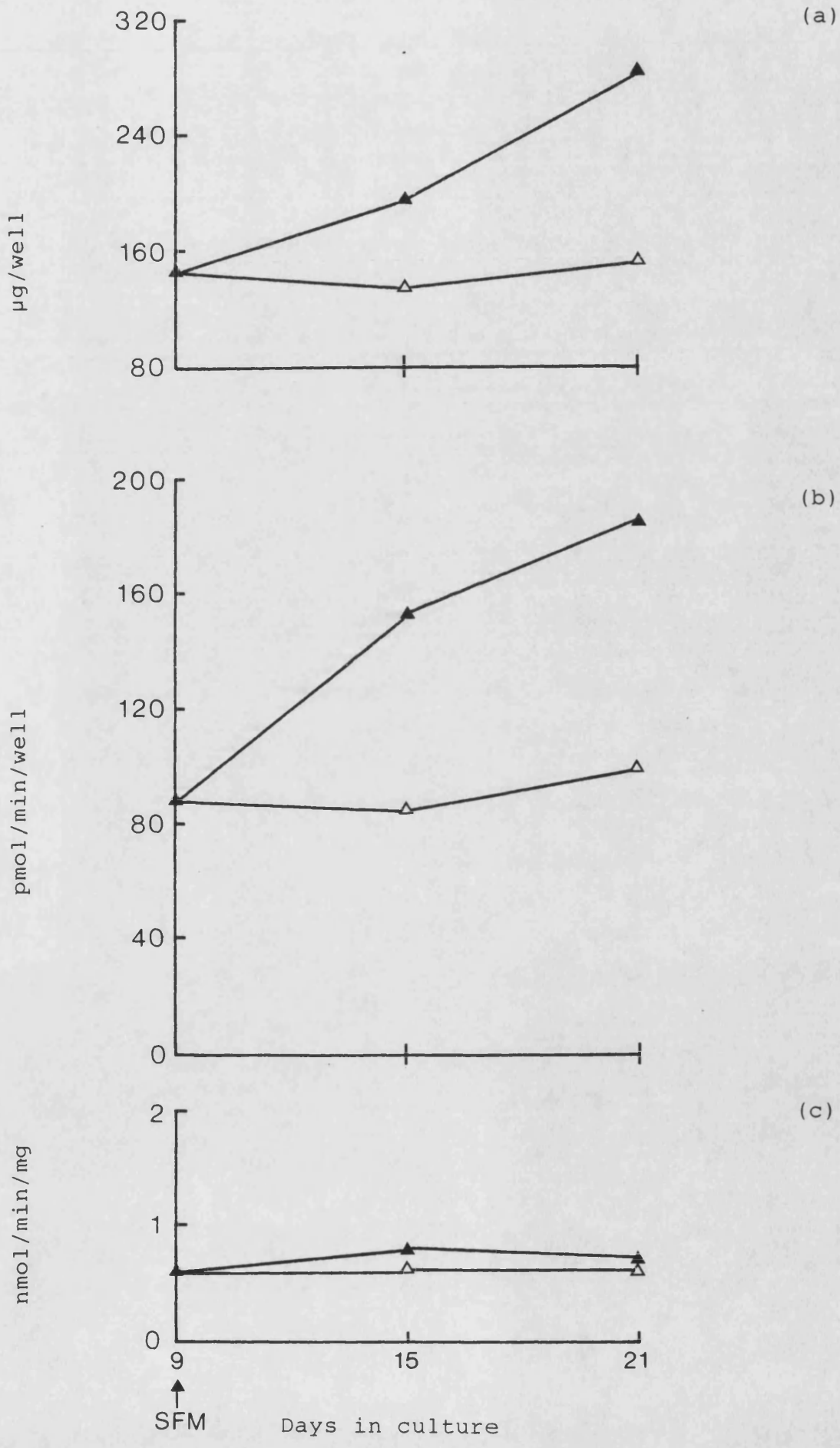
Fig.14 Time course study of the comparative effects on the parameters of foetal human spinal cord neurones grown in serum-supplemented or serum free medium

A 7-8 week old human foetus with intact spinal cord provided 5 million cells on dissociation. Five 4x15mm dia. dishes were seeded at 150,000 cells per cm^2 in HM-A with changes at 3 day intervals. At day 9, one dish was saved for assay and two of the others replenished with SFM (Δ), the others still receiving HM-A (▲).

(a) Protein content per well (μg)

(b) Choline acetyl transferase activity
([^{3}H]-Acetylcholine produced pmoles|min|well)

(c) Specific choline acetyl transferase activity
([^{3}H]-Acetylcholine produced nmoles/min/mg protein)


(a)
320
240
160
80
µg/well
(b)
200
160
120
80
40
0
pmol/min/well
(c)
2
1
0
nmol/min/mg
9
15
21
SFM
Days in culture

rat cultures (see Sections 3.1.8, 3.1.9, 3.1.10,3.2.3,3.2.5).

3.1.7 (iv) FOETAL HUMAN SPINAL CORD-SKELETAL MUSCLE CO-CULTURE

Some successful co-cultures were produced by the deliberate inclusion of a small amount (approx. $2mm^3$) of autologous skeletal muscle tissue in the spinal cord dissociation procedure (Section 2.3.1.5).In addition, a few successful co-cultures were obtained when separately dissociated skeletal muscle cells were added to spinal cord cells in the ratio 1:10 respectively.

Another critical factor was the stage at which the medium was changed from SSM to SFM. If it was changed too early, the muscle cells did not survive- too late and the neuronal clusters were overwhelmed and destroyed. This point could occur anytime between days 3 and 6.

In successful cultures, neuronal clusters could be seen in close proximity to well-developed myotubes. Fine processes could be seen to connect the neuronal clusters with the myotubes in apparent innervation (see Plate 20). These combined structures remained 'healthy' for up to 30 days in SFM and twitching activity was seen in the myotubes at around day 15. This was in marked contrast to neuronal cultures grown alone in SFM, which did not survive beyond day 9 (when transferred to SFM on day 3).

A time course study was performed for protein and choline acetyltransferase activity levels. The mixed spinal cord/muscle cell suspension was seeded at 100,000 per cm^2 in 15mm dia. wells in HM-A. The medium was changed

to SFM on day 4 and every 4 days thereafter. Six wells were harvested, pooled, and assayed, every 4 days. A time course study performed on a pure spinal cord cell culture by the identical procedure is included in Fig.15 (p 191) for comparison.

There appears to be a considerable difference in protein levels (as would be expected with dividing myoblasts and myotubes present), and choline acetyl-transferase activity is 4-fold higher in the co-culture.

3.1.8 LOCALISATION OF ACETYLCHOLINESTERASE ACTIVITY IN THE CULTURES

Dense brown staining was seen in test cultures, concentrated in the neuronal clusters. Control cultures which received, in addition, the acetylcholinesterase inhibitor neostigmine bromide, did not show any such colouration (see Plate 11 (rat) and Plate 16 (human)).

These results demonstrate intense cholinergic activity in the cultures and backs up the high levels of choline acetyltransferase activity seen in the radiometric assays.

3.1.9 LOCALISATION OF TETANUS TOXIN BINDING TO THE CULTURES

The clusters and processes of live cultures (both rat and human) were stained overall, thus helping to confirm their neuronal identity. All controls were negative (see Plate 2 (rat) and Plate 17 (human)).

In fixed cultures there was general intracellular staining throughout, indicating cross-reactivity with

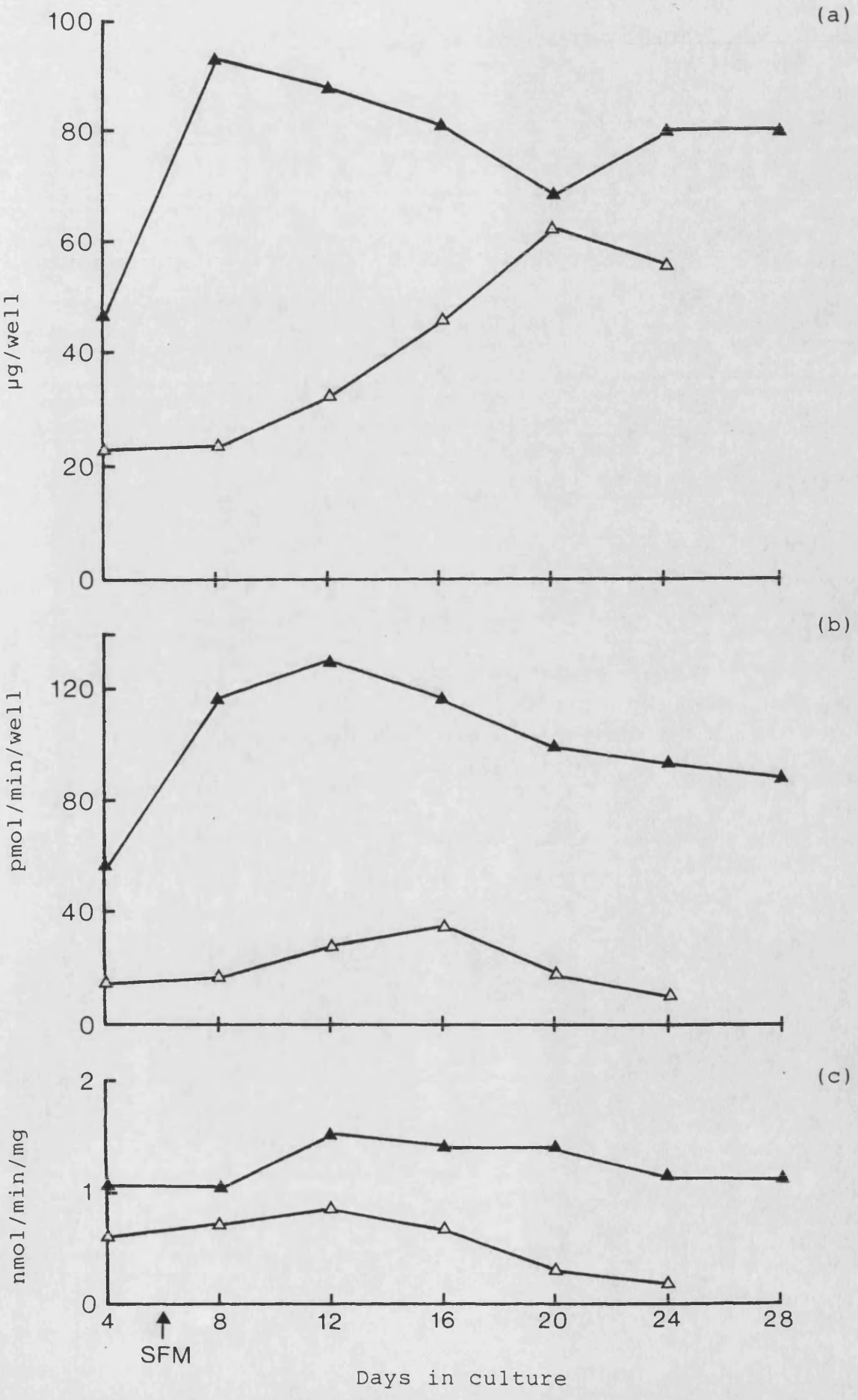
Fig. 15 Time course study of the effect of co-culture of foetal human spinal cord cells and skeletal muscle cells on the culture parameters

Spinal cord cells were seeded in HM-A alone (Δ), or in combination with skeletal muscle cells (▲) at 100,000/cm².
Each point is the result from pooled homogenate of 4 x 15mm dia. wells saved frozen every 4 days.
Assays were performed simultaneously on day 28.

(a) Protein content per well (μg).

(b) Choline acetyltransferase activity
([^{3}H]-Acetyylcholine produced pmoles|min|well).

(c) Specific choline acetyltransferase activity
([^{3}H]-Acetylcholine produced nmoles/min/mg protein).


(a)
100
80
60
40
20
0
µg/well
(b)
120
80
40
0
pmol/min/well
(c)
2
1
0
nmol/min/mg
4
8
12
16
20
24
28
SFM
Days in culture

internal elements (control normal rabbit serum was negative in this respect).

The surface staining produced by tetanus toxin appeared to increase in intensity in relation to the age of the cultures tested, when grown in SFM.

3.1.10 SCREENING OF MND SERA FOR ANTI-NEURONAL ANTIBODIES

3.1.10(i) IMMUNOFLUORESCENT STAINING PATTERNS OF CULTURED CELLS

The cultures were grown on multitest slides by the reported procedure (Section 3.1.2) until day 15-18. They were either used live to demonstrate cell-surface antibody binding, or fixed with 5% acetic acid in 70% ethanol to reveal intra-cellular antibody-antigen recognition. The procedures are identical to those for the indirect immunofluorescence assay to screen hybridoma supernatants (Section 2.3.2.5(i)). The test serum was normally applied diluted 1:10, although other dilutions between neat and 1:100 were also tested on some occassions. The second antibody used was anti-human polyvalent immunoglobulins-FITC conjugate or a mixture of anti-human IgG/IgM-FITC conjugate.

When tested on fixed rat cultures, some staining was always seen. It was possible to delineate three main staining patterns based on the type of cells which were predominantly stained. The most frequent pattern seen, TYPE I, was staining of the neuronal clusters and processes, with only weak, or absent staining of

other cells. TYPE II was staining of background, glial-like cells, and TYPE III was staining of cell nuclei. The comparative results obtained with MND and control sera are summarised in Table 8 p 195 (see also Plate 12).

It is interesting to note that the three main types of staining seen with sera correspond to the patterns seen when supernatants from hybridoma cells produced by fusion of X-63s with spleen cells from mice immunised with spinal cord cell cultures are tested (Section 3.2.3).

The results show that there is no obvious difference in staining between MND and control sera. Both groups of sera produced TYPE I staining at a similar frequency and it was impossible to determine any difference in strength of staining between them, based on this method.

When tested on live cultures, all sera from both groups produced weak-moderate staining. The staining was restricted soleley to the neuronal clusters and processes. No consistent difference could be discerned between the two groups of sera by this method.

Concentration of the immunoglobulin fraction (to 20mg protein/ml) by the ammonium sulphate precipitation method, produced surface staining strong enough for photography (Plate 13) when these samples were tested on live rat cultures.

A few comparative MND/control serum samples were also tested on live and fixed human cultures when available. The results were in line with those seen with rat cultures, and was therefore not systematically pursued.

Table 8 All sera were tested at 1:10 dilution on rat spinal cord cell cultures (fixed in 5%HAc/70%EtOH). Examples of the three different staining patterns seen are found in Plate 12.

	Staining type I	Staining type II	Staining type III
Motor neurone disease sera (47)	36	3	8
Control sera (46)	42	-	4

3.1.10(ii) DETECTION OF IMMUNOGLOBULIN BINDING TO CULTURED CELLS BY ELISA

The cultures were grown in 15/16mm dia. culture dish wells, or many small cultures were produced in 96-well flat bottomed trays. The cultures were used either live or fixed, although the emphasis was on live cultures as the demonstration of cell-surface antibody binding was judged to be the most important. The cultures were pre-incubated with 5% SSM and then the test human sera were introduced to this medium at a final dilution of 1:40 (5µl serum per 200µl medium in an 8mm dia. well). The incubation was for 2/3 h and then the medium was carefully changed several times to remove excess human serum. The second antibody incubation was performed with anti-human polyvalent immunoglobulins-peroxidase conjugate (1:500 in 5% SSM) or alternatively an anti-human IgG and/or IgM specific peroxidase conjugated antisera. The ELISA was completed by the standard method as previously described (Section 2.3.212). Results could be related day to day by the inclusion of a pooled control serum sample as reference.

The cumulated results obtained from these experiments are presented in Table 9 (p 197). The results show no difference except under one set of conditions- when the sera were tested on live rat spinal cord cell cultures, and the second antibody used was an anti-human IgM (µ-chain specific)-peroxidase conjugate. In this case there was an obvious elevation of the mean binding index in the MND group.

Table 9 Comparison of MND and control serum immunoglobulin binding to rat spinal cord cell cultures by ELISA

Culture	Second Antibody	MND sera	Control sera
Rat,live (day 15,SFM)	polyvalent anti-human immunoglobulin	n=28 $\bar{x}$=0.332 SEM=0.019	n=30 $\bar{x}$ =0.358 SEM=0.019
Rat,live (day 18,SFM)	As above	n=31 $\bar{x}$=0.47 SEM=0.02	n=14 $\bar{x}$=0.40 SEM=0.03
Rat, live (day 15, SSM 5%)	As above	n=41 $\bar{x}$=0.58 SEM=0.03	n=39 $\bar{x}$= 0.55 SEM=0.03
Rat,fixed (day 15,SFM)	As above	n=23 $\bar{x}$=0.79 SEM=0.04	n=23 $\bar{x}$=0.71 SEM=0.03
Rat, live (day15, SFM)	Anti-human IgG (γ-chain specific)	n=20 $\bar{x}$=0.23 SEM=0.02	n=20 $\bar{x}$=0.25 SEM=0.02
	Anti-human IgM (μ-chain specific)	n=20 $\bar{x}$=0.63 SEM=0.03	n=20 $\bar{x}$=0.48 SEM=0.03

n=no. of serum samples tested (at 1:40 dilution)

$\bar{x}$=mean O.D. 450nm

SEM=standard error of the mean

3.1.10(iii) INVESTIGATION OF POSSIBLE CYTOTOXIC EFFECT TOWARDS CULTURED CELLS

Samples of MND or control serum/plasma were removed from deep frozen storage and allowed to defrost. They were centrifuged at 4,000rpm (2600g) and then added to 15-18 day cultures in SFM to a final concentration of 10%. The cultures were observed closely over the next 24h.

The same serum/plasma samples were then heat inactivated by standing in a 56°C water bath for 45 min. They were re-tested on fresh cultures in the same way as before.

Finally, the same samples were re-tested on fresh cultures in the additional presence (or absence) of guinea-pig complement (also added at 10%). Three different types of guinea-pig complement were tested in combination with the serum samples (or alone).

Freshly defrosted control or MND sera did not produce any obvious morphological difference on rat cultures over the first 3h when added at 10-20%. When examined after 24h there was a proliferation of the non-neuronal cells and loss of normal "serum-free" morphology in all cases. No unique cytotoxic effect was noted over this time period in either of the groups.

When the sera were heat inactivated and re-tested as above, the results were identical. When the heat inactivated sera were tested in the presence of 10% guinea-pig complement, the cell clusters became phase-dark within three hours, with some detachment. This indicates

vacuolation and cell death. Unfortunately, this was observed in all cases and when the three different types of complement were added alone. It would thus appear that guinea-pig complement (ie. serum) was itself toxic to rat spinal neurones in culture. A cytotoxicity assay could not be developed under these circumstances.

INTRODUCTION TO RESULTS SECTION 3.2 MONOCLONAL ANTIBODIES

Monoclonal antibodies are powerful tools in neurobiology for identification, assay and developmental studies of specific cell types of the nervous system. At the commencement of this project few were commercially available and the potential for the generation of interesting and useful novel antibodies directed at neural antigens was virtually unlimited. Although the direct goals were the production of an anti-neurofilament, anti-choline acetyltransferase and anti-neuronal membrane monoclonal antibody, other interesting antibodies were also generated by the immunisation procedures employed. The characteristics of the monoclonal antibodies generated during the course of this project are reported in the forthcoming section.

3.2 MONOCLONAL ANTIBODIES

A list of all the monoclonal antibodies, established during this work, together with their properties is given in Table 10 (p 201).

3.2.1 TYPICAL FUSION AND CLONING PROCEDURES

Fusions carried out as described in the Methods Section yielded an average of 50% of the wells bearing hybridoma colonies. The number of these wells which gave positive, or mixed positive results in the relevant initial screening assay varied greatly depending on the antigen used (see Sections 3.2.3,3.2.4,3.2.5).

The first cloning procedure also yielded an average of 50% colony-bearing wells in the plates seeded at 1 cell per well. The plates seeded at 2 and 5 cells per well by limiting dilution were "safety nets" to allow for possible poor viability of some cells.

The number of wells which gave positive results after the first cloning procedure was usually quite small. It did not normally exceed 10% and was usually much lower (0-2%). This presumably resulted from the presence of multiple hybridoma cell lines in the original positive well, and/or positive hybridoma cell reversion to a non-secreting type through initial instability and chromosome loss.

The number of positives obtained after the second cloning was usually 90-100% by micromanipulation and 50-100% by limiting dilution. As would be expected these figures indicate that it is safe to assume that the

Table 10 Monoclonal Antibodies Produced N/T = not tested

Immunisation	Fusion no.	Ab code	Times cloned	Ig class	Immunofluorescent staining pattern		Specificity (?=suspected)		Human culture cross-reactivity	Immunoblot bands	Other characteristics
					fixed cells	live cells	cell	antigen			
Foetal rat spinal cord cell culture	1	1C3	x3	IgG2a	nuclei (homogneous)	none	non-neuronal?	nucleoskeletal protein?	yes	40KDa?	-
		2F7	x4	IgG2b	cell clusters & processes	none	neurones	neurofilament protein	yes	200K 160K	less specific with acetone fixation Occ.weak nuclear stain
		2F3	x1	N/T	nuclei (envelope?	none	-	-	N/T	N/T	-
Bovine choline acetyltransferase	1	3H9 3C11 2B6 4H4 1C7 5B9 3A8 5C7	-	N/T	none	none	-	non-cross reactive or contaminant	N/T	N/T	all very strong positive response with initial immuno-dot binding screen

Table 10 (continued)

Immunisation	Fusion no.	Ab code	Times cloned	Ig class	Immunofluorescent staining pattern		Specificity (?=suspected)		Human culture cross-reactivity	Immunoblot bands	Other characteristics
					fixed cells	live cells	cell	antigen			
Bovine ChAT	2	2F1	x2	N/T	none	none	-	-	N/T	N/T	strong immuno-dot +ve
		3G6	x2	N/T	"bipolar" cell	none	schwann?	cont-aminant	N/T	-	"
P3 synaptic membrane pellet	1	5A5	x3	IgM	background cells	none	glial?	GFAP?	yes	50KDa?	stronger stain with acetone fix
	3	1B7	x2	N/T	general	small round cells	glial?	?	yes	-	cells develop fine processes in older cultures
	5	3C4a	x4	IgM	-	clusters & proc-esses	neurones ?	surface glyco-protein	yes	-	serum inducible trypsin-sens. Insol. in org. solv.
		3C4b			background cells	-	fibro-blast	cytoskel-etal protein	yes	N/T	"-ve" staining effect

hybridoma cell lines established through micromanipulation are now derived from a single cell and are hence producing monoclonal antibodies. In practice, however, these cell lines were often cloned a third time by either method before bulk growth or frozen storage.

The positive cells cloned by limiting dilution, however, were always cloned a third (and sometimes fourth) time until their positive ratio was greater than 90%.

Cell lines removed from frozen storage were usually re-cloned prior to bulk growth.

3.2.2 EXPANSION OF CLONAL HYBRIDOMAS IN TISSUE CULTURE

It proved wise to gradually expand the cells into larger scale culture. They were sequentially moved from 15/16mm dia. wells in 1ml media to 35mm dia. dishes in 3ml media, then 50ml "T" flasks in 10ml media etc. A sudden change to large volume often resulted in cell death and complete loss. In addition, some cell lines appeared to retain a dependence on macrophages or macrophage-conditioned medium for their survival and could be lost if this was not provided.

Sub-culturing was performed by resuspending a confluent monolayer of cells in a 50ml "T" flask into the supernatant (15ml). This suspension (approx. 13ml) was then withdrawn and gently centrifuged in a 30ml Sterilin container. The antibody-containing supernatant was transferred to a second Sterilin tube and stored at 40°C. The cells could either be discarded or resuspended and stored frozen. The "T" flask was re-filled with

fresh medium (13ml) and replaced in the incubator. As with the myeloma cell line, it was usually necessary to carry out this procedure every 2 or 3 days. The percentage of cells secreting antibody declined with many sub-culturings (as demonstrated by the direct immunofluorescence method (2.3.2.7) and it was necessary to frequently re-clone the cells or remove fresh from frozen storage.

3.2.3 HYBRIDOMA CELL LINES ESTABLISHED FOLLOWING IMMUNISATION WITH FOETAL RAT SPINAL CORD CELL CULTURES

Mice were immunised and their spleen cells fused with myeloma cells as described in the Methods Sections (2.3.2.1 to 2.2.2.4).

First batch of cells One fifth of the cells from the fusion were initially plated out (the rest were stored frozen). In this plate, colonies were seen in 58 (60%) of the wells. When tested by the indirect immunofluorescence screening procedure, 28 (48%) of these colony supernatants produced intra-cellular staining of the foetal rat spinal cord cell cultures. Unfortunately, in almost all cases, this staining was uniform suggesting multiple hybridoma colonies in each well or (less likely) a single hybridoma colony producing antibody cross-reactive with many antigens. There was only one example of less general staining (well C3) and this was further investigated in detail.

The specific staining noted appeared to be directed against the nucleus of the large, flat fibroblast-like cells.

Subsequent cloning by micromanipulation yielded 48 (from 192) colonies 100% of which were positive by the indirect immunofluorescence test.

A further cloning procedure by micromanipulation yielded 104 (from 192) colonies 100% of which were positive.

The immunofluorescent staining patterns were more fully examined with culture supernatant from these cells. It was noted that not only were the large, oval nuclei of the flat fibroblast-like background cells stained (fibroblasts and Type I astrocytes), but also the smaller, rounder nuclei of cells which were closely associated with the neuronal clusters and processes (presumably Type II astrocytes). It was difficult to see through this staining into the three-dimensional neuronal clusters, but it did appear that underlying cells were not stained (see Plate 4). This may, however, have been an artefact of poor penetration of the antibody into the cluster.

When tested on human spinal cord cultures the monoclonal antibody supernatants produced the same nuclear staining pattern thus demonstrating inter-species cross-reactivity (see Plate 17).

Second batch of cells A second sample (1/5 original) of the fusion cells was defrosted and plated out. Colonies were produced in 42 (44%) of the wells.

On screening, two examples of immunofluorescent staining restricted to the neuronal clusters and processes were seen. The staining appeared identical to that observed with the donated anti-neurofilament monoclonal antibody (John Wood, RT97) (see Plate 1).

One of these (2F7) was cloned, producing 11 (from 92) positives in the first cloning. The fastest growing, strongest positive antibody producing hybridomas were pursued through a further three clonings before bulk growth.

The immunoglobulin class was shown to be IgG2b and immunoblot analysis using adult rat spinal cord homogenate as antigen showed strong affinity for two bands at approximately 200KDa and 160KDa (but not 70KDa). This corresponds with the Mwts of the two main polypeptides of the neurofilament triplet (see also Sections 3.2.8 and plate

The antibody produced a similar staining pattern in human spinal cord cell cultures (see Plate 17) and was therefore used regularly as a marker for neurones in both types of culture.

A second example of specific staining was observed in one well (2F3) from this fusion plate. In one respect it was similar to the 1C3 nuclear staining antibody, but on closer examination the staining appeared to be localised to the nuclear envelope (see Plate 5). It was cloned twice and stored frozen.

3.2.4 HYBRIDOMA CELL LINES ESTABLISHED FOLLOWING BOVINE CHOLINE ACETYLTRANSFERASE IMMUNISATION

Mice were immunised with bovine ChAT as described in the Methods section 2.3.2.1. When the pre-fusion sera were tested they gave strong positive reactions in the dot-immunobinding screening assay using ChAT as antigen, at 1:100 dilution. Normal mouse sera produced only a faint response at 1:100 dilution.

When tested on rat spinal cord cell cultures (at 1:10 dilution), both sera produced moderate overall diffuse staining, predominantly of the neuronal clusters and processes. Normal mouse sera did not produce staining discernibly stronger than control background levels.

Two successful fusions (Section 2.3.2.4) were performed with spleen cells from the immunised mice. On initial testing, a total of ten supernatants from both fusions produced strong positive spots in the dot-immunobinding screen for anti-bovine ChAT antibodies. When tested on fixed rat cultures for immunofluorescent staining (Section (Section 2.3.25(i), however, only one of the supernatants produced staining distinguishable from control background levels. The staining produced was not similar to that seen with the sera, but appeared to pick out a few cells with a "bipolar" morphology. While the rest of the hybridomas were stored frozen, the cells from this well (3G6) were cloned twice and the supernatants retested. High power photography under UV illumination (see Plate 6) has demonstrated that the antibody decorates an intracellular cytoplasmic antigen. The nucleus is not stained. The stained cells are "bipolar" and apparently trace the route of processes emanating from the neuronal clusters.

3.2.5 HYBRIDOMA CELL LINES ESTABLISHED FOLLOWING IMMUNISATION WITH SYNAPTIC MEMBRANE FRACTION (P3)

Mice were immunised with synaptic membrane fraction (Section 2.3.2.1) and their spleen cells were fused with mouse myeloma cells as described in the Methods (Section 2.3.2.4).

The immunofluorescence assays performed with hybridoma supernatants from these fusion procedures were modified so that live cultures could be used. This was essential to ensure that the antibodies demonstrated were to cell-surface components. The cultures had to be handled with extreme care, and were used at no more than 15 days old, to minimise detachment from the glass surfaces. In addition, all incubations and washing was carried out in SSM,as BSS ± 5%NRS appeared to greatly increase detachment. All tests were made in duplicate. All other times and conditions were identical to those for fixed cultures.(Section 2.3.2.5(i)).

When the pre-fusion sera were tested by the above method on live rat spinal cord cell cultures, they produced overall moderate staining of the cell surfaces at 1:10 dilution. Normal mouse serum did not produce staining discernibly stronger than control background levels under these same conditions.

From five fusions, approximately one thousand supernatants from hybridoma containing wells were tested in total on live rat cultures in a search for cell-surface specific antibodies. Many were also tested on fixed cultures.

In the first fusion no positives were seen when tested on live cultures. Of the few that were also tested on fixed cultures, however, there were some examples of intracellular staining. One in particular (5A5) strongly stained star-like glial cells (Type II astrocytes) and other background cells (see Plate 3). It was cloned

three times. The immunoglobulin class was determined as IgM.

The second fusion did not produce any recognisable positives when supernatants were tested on live cultures.

The third fusion did, however, give rise to one well (1B7) where supernatant gave a definite staining pattern when tested on live cultures. It may be useful to note that the cultures used in the initial screening procedure were younger than usual (4-7 days). The staining was not, unfortunately, specific to the surface of the neuronal clusters and processes, but appeared to pick out a number of very small, round, non-process bearing cells which were mainly associated with the clusters (see Plate 7). When tested on older cultures, a few cells were still weakly stained. They appeared to be embedded in the clusters and had now taken on a more irregular, fine-process bearing morphology (see Plate 7). Their nature is uncertain but it seems likely that they are a type of glial cell, or glial cell precursor. The hybridoma cells were cloned twice and stored frozen.

The fourth fusion gave a few wells with supernatants of similar specificites to those described above from fusion three. There was, however, no well showing staining of the surfaces of neuronal cluster and processes.

One well (3C4) from fusion five yielded the first example of the latter staining pattern. The staining was very weak but clearly distinguishable from controls. When tested on fixed cultures it produced a very strong overall stain of clusters, processes and background

cells. The first cloning of the cells (by limiting dilution) gave ten wells showing the weak surface staining described above. All ten supernatants also showed the intra-cellular staining pattern. One of the supernatants was tested against a four day old human culture and produced the strongest example of surface staining seen to this point (see Plate 18). The hybridomas from the ten positive wells were transferred to 15mm dia. wells and allowed to expand. Eight were frozen down and the other two re-cloned (again by limiting dilution).

The supernatants from the larger wells were tested on a 12 day old human culture, Some of which had been transferred to SFM on day 6 and others maintained in SSM. The difference in staining strength between the two sets of cultures was obvious and consistent. In all cases the cultures maintained in SSM stained strongly, while those in SFM were only weakly stained.

The surface antigen, recognised by the above supernatant, was apparently associated with cells grown in serum. The disappearance of this antigen during culture in serum free medium was monitored by immunofluorescence, using rat cultures transferred to SFM at day 3, compared to those maintained throughout in SFM and 5% serum (DHS and FCS). The same supernatant was used for comparative tests, performed at three day intervals. By day 15, the staining of cultures grown in SFM had declined to levels that were not easily distinguishable from controls. The strength of staining produced in the serum-supplemented medium also declined, but to a much lesser degree. It

was still clearly distinguishable by day 15. By days 18-21 there was some interfernce from the still-dividing non-neuronal cell types.

The phenomenom of serum inducibility was further pursued by an ELISA method (Section 3.2.9).

The hybridomas tested above were cloned for a second time when several wells gave supernatants which showed strong surface staining of cultured rat cells. The best were transferred to 15mm dia. wells.

It was noticed that some of the supernatants from the successfully expanded hybridomas did not produce a strong background stain when tested on fixed cultures- only the clusters and processes were stained. In contrast, other supernatants only produced a strong staining of background cells and the clusters and processes were completely untouched creating a striking "negative" type effect. Dark, unstained neuronal processes could be clearly seen to run over the surface of the stained cells (see Plates 9 and 10). it was suspected from these observations that the surface and intra-cellular staining were attributable to separate antibodies derived from two different hybridoma cell lines. These were named 3C4a and 3C4b respectively as they had originated from the same fusion well.

The striking pattern produced in fixed cultures by 3C4b was not similar to that produced by 5A5 (suspected anti-GFAP) in that there was no staining of the star-like Type II astrocytes which predominantly organise themselves in close contact with the neuronal clusters and processes.

Only large, flat fibroblast-like cells were stained. With acetone fixation the staining was intensified and fine networks of intra-cellular filaments could be clearly visualised within these cells (see Plate 10).

The results of experiments designed to investigate the nature of the 3C4a surface antigen are reported in the relevant forthcoming Sections (3.2.7, 3.2.8, 3.2.9).

3.2.6 DIRECT DEMONSTRATION OF IMMUNOGLOBULIN PRODUCTION BY HYBRIDOMAS

Immunoglobulin production of established hybridoma cell lines was followed by the procedure outlined in Section 2.3.2.7.

In tests where the cells were fixed prior to incubation with the anti-mouse Ig-FITC conjugate, the vast majority of cells were homogeneously stained (see Plate 21). This did not appear to diminish greatly after several sub-culturings.

When the cells were tested live, however, there was a marked decrease in the number of cells which were demonstrated to be secreting immunoglobulin as shown by irregular surface staining patterns (see Plate 21).

It would, therefore, appear that although the cells retain intra-cellular immunoglobulin, their capability to secrete it continually declines.

The class of immunoglobulin secreted by the different hybridomas could be determined by the method described in Section 2.3.2.10.These determinations are shown in Table10 (p201).

3.2.7 IMMUNOFLUORESCENT STAINING PATTERNS

The pattern of staining produced by each monoclonal antibody was carefully examined, often at different stages of culture development and under varying medium conditions. The method used to study antibodies recognising intra-cellular antigens was identical to the basic screening procedure (Section 2.3.5.1(i)). In addition, alternative fixation methods were tested to see if the characteristic immunofluorescent staining patterns were affected.

When acetone was used, only cultures grown on glass coverslips or 10-well multitest slides could be used. They were rinsed and then immersed in the acetone at -20°C for 10 min. They were air-dried prior to further processing by the standard method (Section 2.3.2.5(i)).

Cultures could also be fixed by exposure to 4% paraformaldehyde (w/v) in PBS for 15h at 4°C. They were rinsed with PBS alone before further processing by the basic method (Section 2.3.2.5(i)).

The paraformaldehyde method produced poor, indistinct results with all antibodies tested (results not shown).

The acetone fixation method affected the staining characteristics of three of the monoclonal antibodies. The 2F7 (anti-neurofilament) monoclonal antibody staining appeared to become less specific with greater background staining of non-neuronal cells and nuclei. The 5A5 (possible anti-GFAP) monoclonal antibody staining became greatly intensified, with more definite staining of

large, flat fibroblast-like cells (Type I astrocytes). The 3C4b (intracellular) monoclonal antibody staining was particularly affected by acetone fixation. It became far more intense and distinct so that individual intracellular filaments could be distinguished in some large, flat fibroblast-like cells (see Plate 10(C)). In addition, a second type of staining could be distinguished (in cells with similar morphology) where there was more diffuse overall staining of the cytoplasm, with fine "lace-like" edges visible (see Plate 10(B)).

3.2.8 WESTERN BLOT ANALYSIS OF MONOCLONAL ANTIBODIES

All established monoclonal antibodies were examined by Western Blot analysis using spinal cord homogenate, culture homogenate or P3 membrane pellet as antigen. Only the 2F7 anti-neurofilament monoclonal antibody produced a consistently positive result (see Plate 22). It recognised two bands of approximately 200KDa and 160KDa, recognising an antigenic determinant common to two of the neurofilament polypeptide triplet. No band was ever seen in the region of 70KDa, the molecular weight of the third polypeptide.

A weak response which was not visible after photography was also seen with some of the other monoclonal antibodies(1B6,5A5,1C3). Unfortunately, no band was ever seen on the several occasions that the 3C4a surface staining antibody was tested. The 3C4b intracellular antibody was not tested at this time.

3.2.9 ENZYME-LINKED IMMUNOSORBENT ASSAY ANALYSIS OF MONOCLONAL ANTIBODIES

Established monoclonal antibodies (Table 10, p201) were examined by ELISA (Section 2.3.2.12) using rat spinal cord cell cultures as antigen.

In fixed cultures, the 2F7 anti-neurofilament monoclonal antibody gave a strong signal which was proportional to the quantity of neurofilament protein present. The results obtained have been presented in detail in previous Sections (3.1.4, 3.1.5, 3.1.6).

The 5A5 and 3C4b monoclonal antibodies also produced strong signals when tested on fixed cultures, but were not used in semi-quantitative comparative assays.

The 3C4a surface staining monoclonal antibody produced a good signal when tested on live cultures, and this method was effective in providing a comparative time-course study of the binding of the antibody to cultures grown either in serum-supplemented or serum-free medium (Fig. 16, p 216). The graph shows the clear difference in strength of binding between the two and confirms the observed difference seen with the immunofluorescence technique (Section 3.2.5).

When homogenates of cultures, adult rat spinal cord and P3 membrane preparations were used as antigen (Section 2.3.2.12) in the ELISA, the 3C4a antibody again produced a good signal. It was also noted that the 5A5 and 2F7 antibodies gave strong signals, notably, even with the P3 synaptic membrane pellet (Table 11, p218).

The sensitivity of the antigen(s) to organic

Fig. 16 Time course ELISA of 3C4a/b antibody binding to live rat spinal cord cell cultures grown in SFM or SSM

The ELISAs were performed at three day intervals using the same sample of antibody supernatants under identical conditions. Each point for the 3C4a/b antibody is the average of two wells. Each point for the 5A5 antibody (intracellular control) and background control (second antibody only) is the mean of the average of two wells from the SFM and SSM culture.

□ = background control (second antibody alone)

○ = 5A5 antibody binding (intracellular control)

● = 3C4a/b antibody binding (cultures transferred to SFM at day 3)

■ = 3C4a/b antibody binding (cultures transferred to SFM + 5%DHS/FCS at day 3)

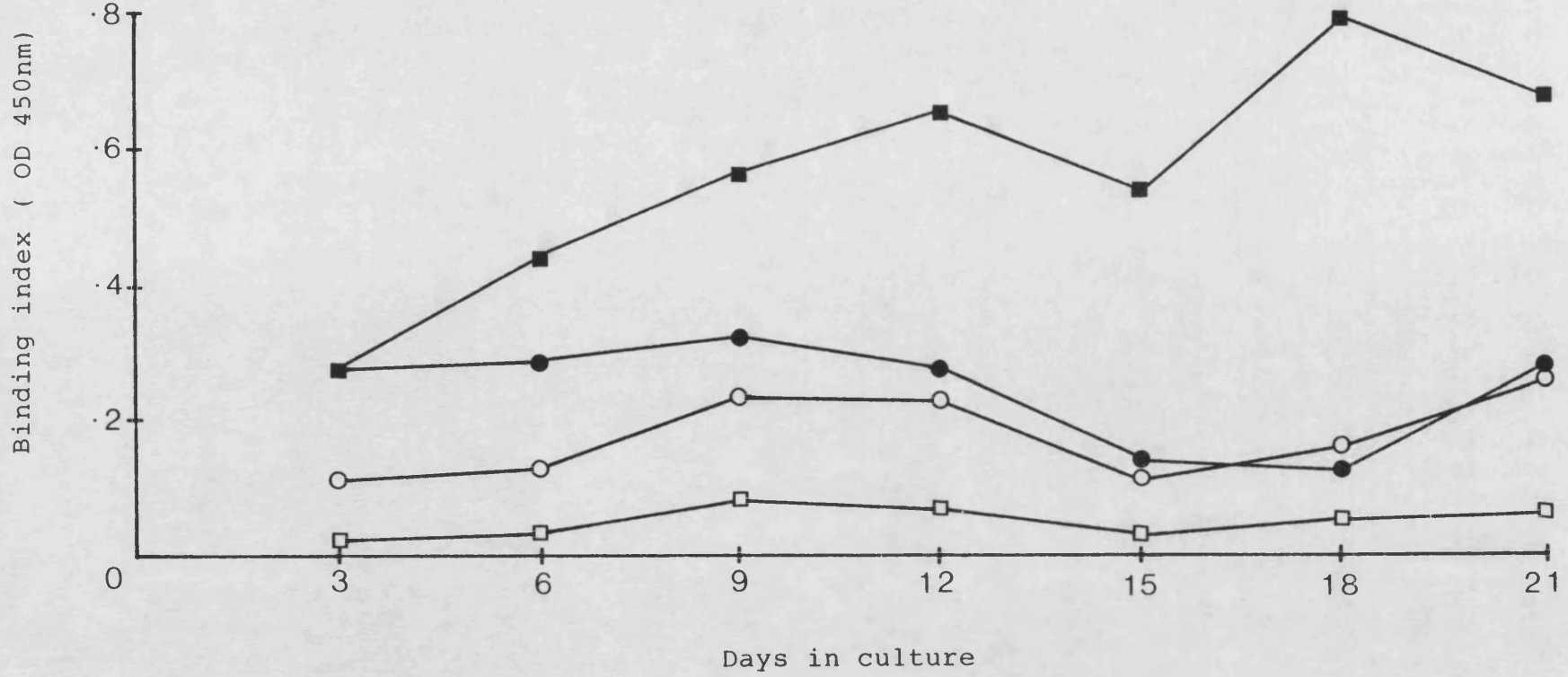

Binding index (OD 450nm)
·8
·6
·4
·2
0
3
6
9
12
15
18
21
Days in culture

Table 11 Reactivity of antibodies with various antigen sources as demonstrated by ELISA.

Antibody / Antigen	O.D. 450nm				
	3C4a/b	3C4a	1B7	5A5	2F7
Adult rat spinal cord homogenate	2.49	1.10	1.78	0.60	2.48
Adult rat spinal cord "P3" membrane fraction	2.35	1.10	1.84	0.43	2.02
Foetal rat spinal cord cell culture homogenate	2.5+	1.42	2.26	0.97	1.19

All antigens were coated at 10µg/ml. The batch of antibody supernatant was the same for each antigen, as were all other conditions. The final incubation was 30min at room temperature.

Note the high content of intracellular antigens still present in the P3 membrane pellet (all but the 3C4a antibody produce intracellular staining in the immunofluorescence test).

solvent was also tested by the ELISA method. To samples of the tissue homogenate were added chloroform, methanol or chloroform/methanol (10 vol.). This was shaken vigorously for a few minutes and then centrifuged (microfuge, high speed). the organic solvent was discarded and the pellet resuspended in carbonate buffer and dispensed into the wells as usual. When the homogenates were extracted as above, there was no difference in response to the 3C4a monoclonal antibody when compared to the un-extracted homogenate (see Table 12, p220).

It was also possible to study the trypsin sensitivity of the coated antigens by interjecting an incubation with 0.05% trypsin solution between coating and the primary antibody incubation. When the plates coated with homogenate were subjected to trypsinisation prior to incubation with 3C4a antibody, the signal was considerably reduced (see Table 13, p 221).

3.2.10 IMMUNOPRECIPITATION

This was used to help clarify whether monoclonal antibodies raised to the choline acetyltransferase preparation were directed towards the enzyme itself, or to some other contaminant present. Alternatively, the monoclonal antibody might not be cross-reactive with rat choline acetyltransferase.

The crude enzyme preparation (100µl) was incubated with suspected anti-choline acetyltransferase monoclonal antibody (150µl), or another control monoclonal antibody (eg. anti-neurofilament) for several hours at 4°C. Goat

Table 12 Effect of extraction with organic solvent on the antigenicity of rat spinal cord homogenate as demonstrated by ELISA.

Antibody / Antigen	O.D. 450nm			
	3C4a	1B7	5A5	2F7
Adult rat spinal cord homogenate	0.48	0.51	1.67	1.85
Chloroform/methanol extracted homogenate	0.51	0.38	1.45	1.46

Both antigen sources were coated at 10µg/ml. Antibody supernatant was from the same batch. All other conditions were identical. Final incubation 30min, room temperature.

Table 13 Effect of incubation with trypsin on the antigenicity of rat spinal cord fractions for the 3C4a antibody.

Antibody / Antigen	O.D. 450nm	
	3C4a No trypsin	3C4a + trypsin
Adult rat spinal cord homogenate	0.47	0.06
Adult rat spinal cord P3 membrane fraction	1.44	<0
Foetal rat spinal cord cell culture homogenate	1.18	0.11

All antigens were coated at 10µg/ml. The results are those obtained after the subtraction of non-specific binding values.

Note the apparent abolition of the antigenicity of the fractions for the 3C4a antibody, following trypsin treatment.

anti-mouse Ig antisera (100µl) was then added and incubated for several hours at 4°C. The samples were then centrifuged (MSE microcentaur, high speed) and aliquots of the supernatant assayed for residual choline acetyltransferase activity by the standard method (Section 2.3.1.10). On no occassion was the level of choline acetyltransferase activity reduced with comparison with the control thus helping to confirm that the monoclonal antibodies raised against the crude commercial preparation do not appear to be directed at the enzyme itself.

PHOTOGRAPHIC PLATES

1 - 22

PLATE 1

A, B and C are the same rat spinal cord cell culture (day 18) grown by the standard method (Section 2.3.1.6) from day 3 in SFM. It has been fixed with 5%HAc/70% EtOH and processed for indirect immunofluorescence (Section 2.3.2.5(i)) using the 2F7 monoclonal antibody (see Table 10, p201).

A. Large cell cluster and processes (LP x 100, Tungsten lamp).

B. Same field of view as in A (above). The 2F7 monoclonal antibody has specifically stained the cell cluster and processes. Similar results (not shown) are seen with the RT97 anti-neurofilament monoclonal antibody (Wood & Anderton, 1981).

C. Single neuronal process (HP x 400, UV lamp).

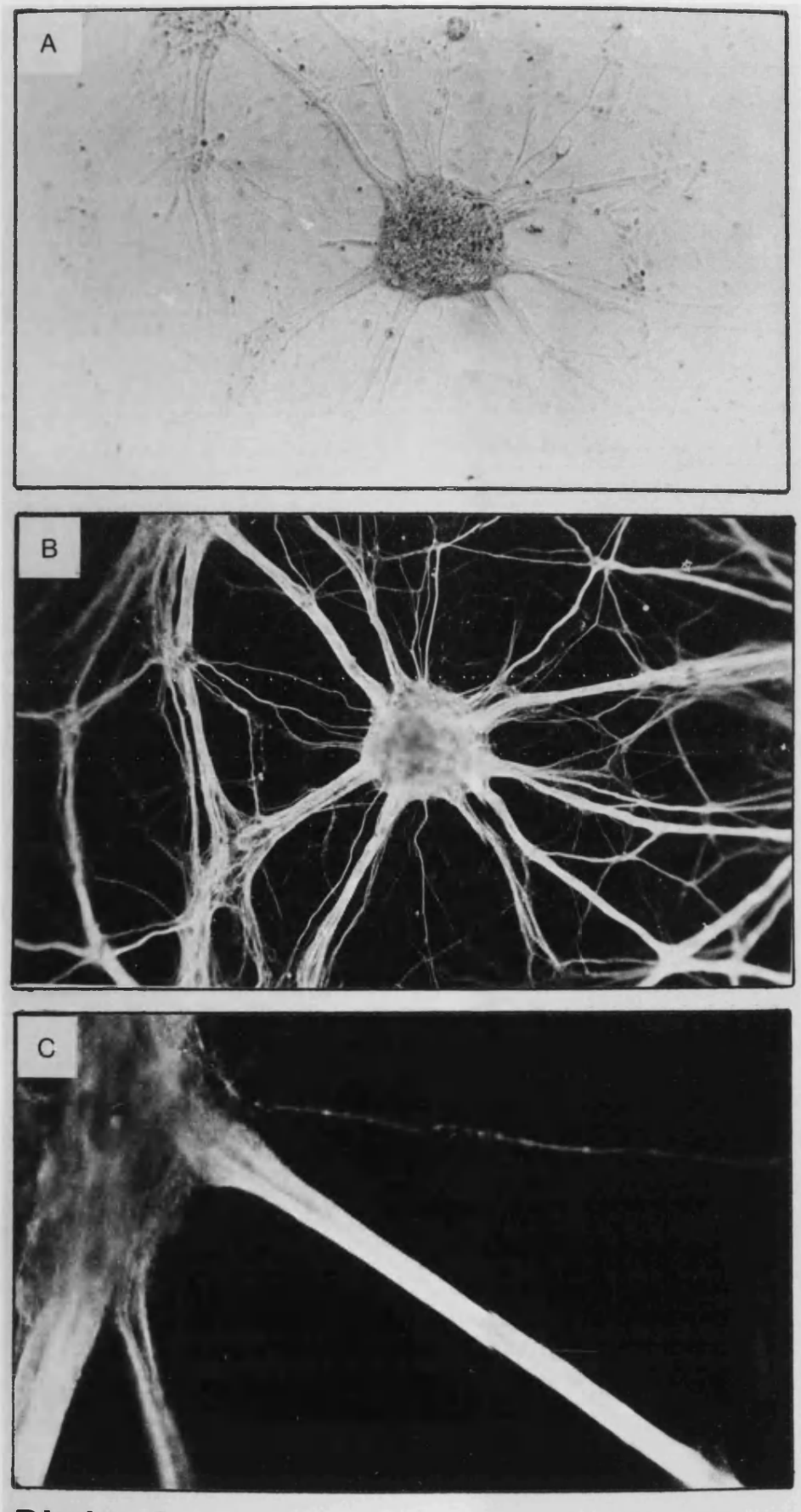


Plate 1

PLATE 2

A, B and C are the same rat spinal cord cell culture (day 18) grown by the standard method (Section 2.3.1.6) from day 3 in SFM. The culture has been processed for indiect immunofluorescence (Section 2.3.2.5(i)), without prior fixation, using tetanus toxin and antiserum (Section 2.3.1.13).

A. Large cell clusters and processes (LP x 100, Tungsten lamp).

B. Same field of view as in A (above). The tetanus toxin has specifically stained the cell clusters and processes, thus confirming (in conjunction with anti-neurofilament monoclonal antibody binding; Plate 1) their neuronal identity (LP x 100, UV lamp).

C. A high power photograph of B (above) showing the diffuse nature of tetanus toxin binding to a single neuronal process (HP x 400, UV lamp).

The overall staining produced when tetanus toxin is tested on fixed cultures is not shown, but it is similar to that seen in Plate 9(B).

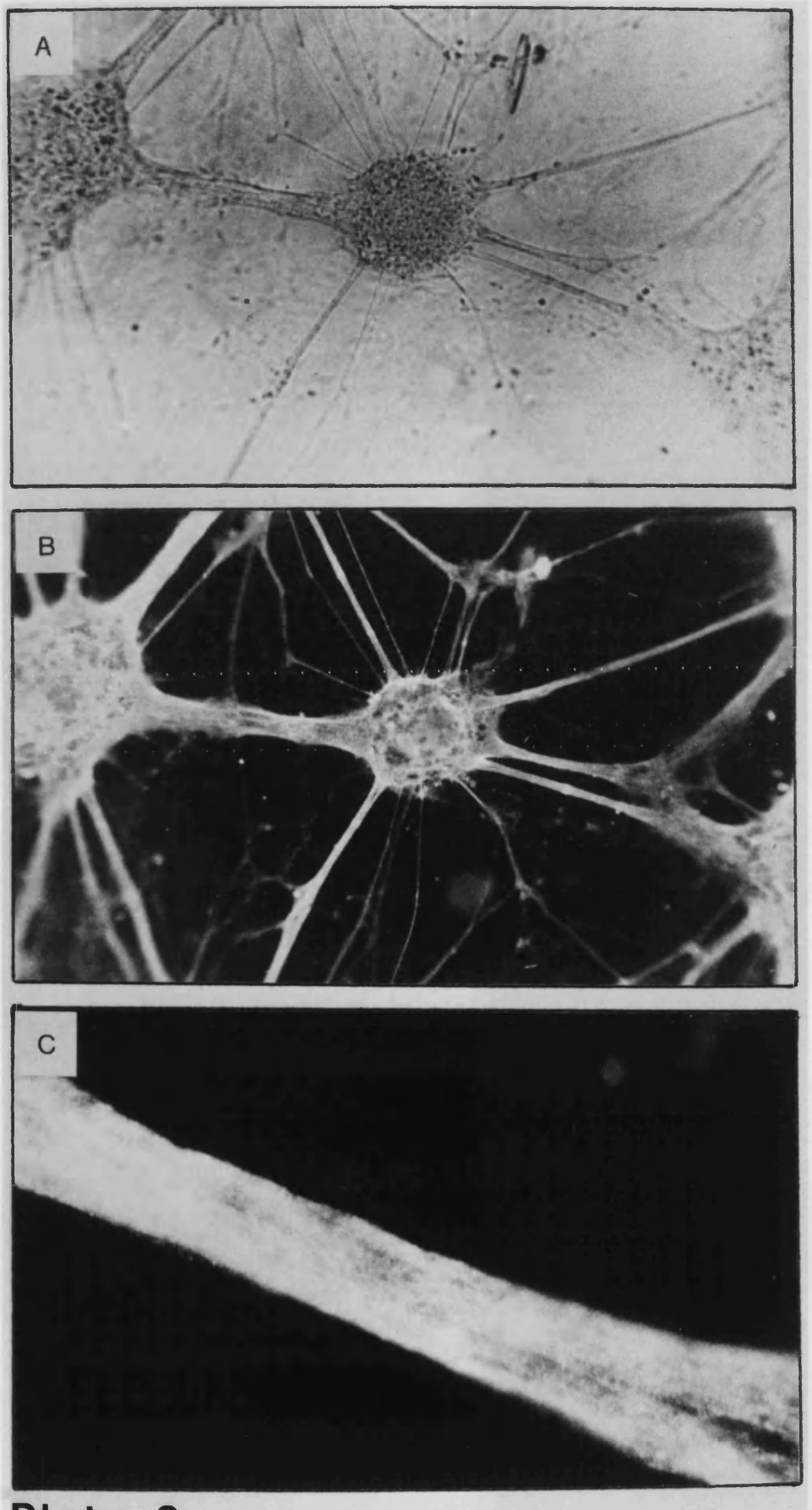


Plate 2

PLATE 3

A, B and C are the same rat spinal cord cell culture (approximately day 12). The culture was prepared by the basic method (Section 2.3.1.6) except it was allowed to remain in SSM for longer (day 4/5) thus producing an "impure" culture. The culture was fixed in 5% HAc/70% EtOH and processed by the indirect immunofluorescence technique (Section 2.3.2.5(i)) using the 5A5 monoclonal antibody (see Table 10 p201).

A. This shows the impure nature of the culture, with the neuronal cluster and processes partially obscured by non-neuronal cells (LP x 100, Tungsten lamp).

B. Star-like astrocytes are stained strongly. They appear mostly in close association with the neuronal cluster and processes (LP x 100, UV lamp).

C. This is an enlargement of a section of photograph B (above). A well-defined Type II astrocyte is clearly depicted in the central section (3½ x enlargement, UV lamp).

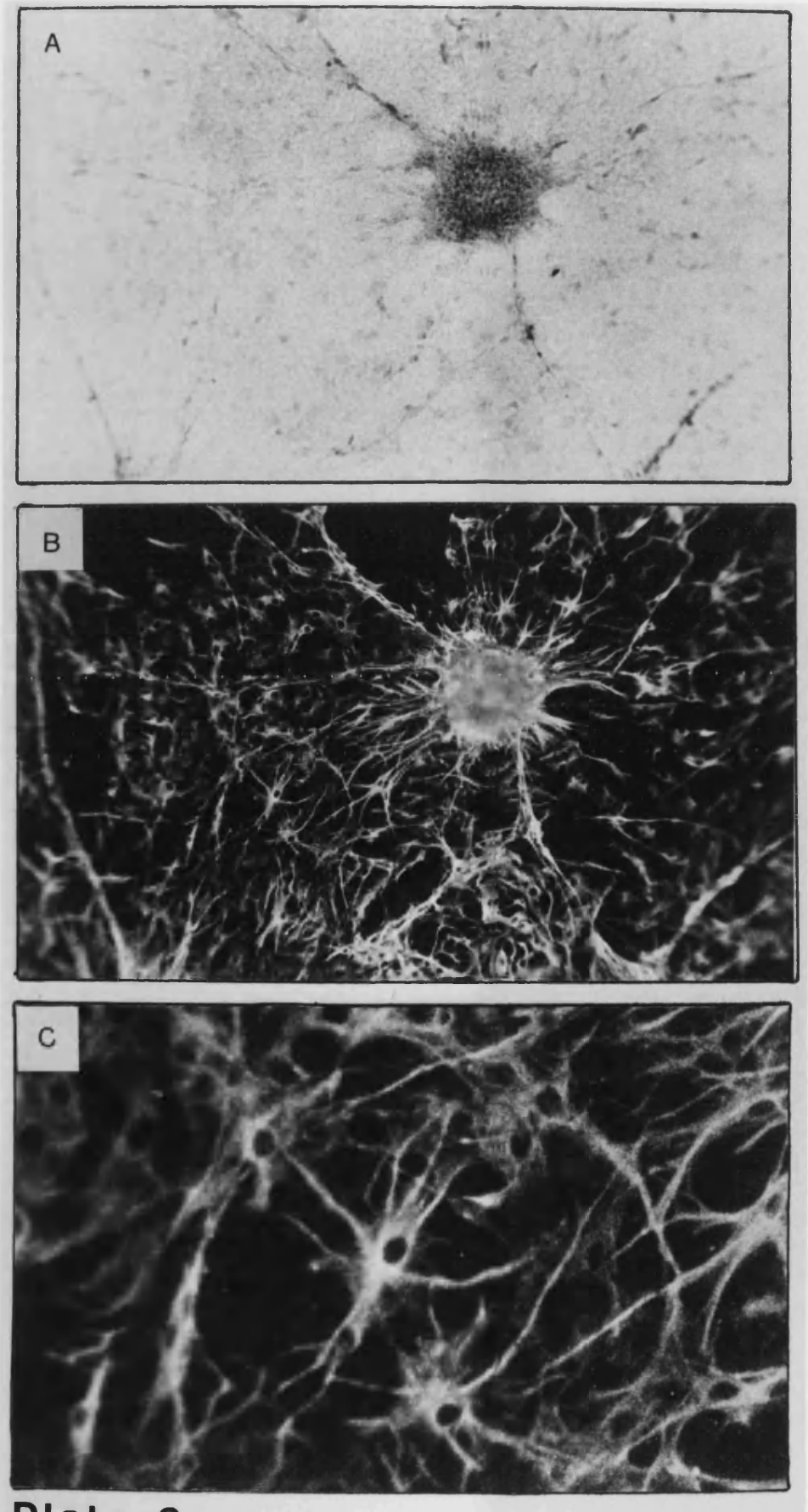


Plate 3

PLATE 4

A and B/C are both rat spinal cord cell cultures (15-18 days) which have been fixed in 5%HAc/70% EtOH and processed by the indirect immunofluorescence method (Section 2.3.2.5(i)) using the 1C3 monoclonal antibody (see Table 10 p 201).

A. This culture was grown by the basic method (Section 2.3.1.6) in SFM from day 3 with the additional inclusion of the mitotic inhibitors uridine (3.5µg/ml) and deoxyfluorouridine (1.5µg/ml) added to the SFM at day 3 only. Note the staining of small, roundish nuclei surrounding the neuronal clusters. The sparse staining of large oval background cell nuclei gives an indication of the neuronal purity of the culture obtained by this method (LP x 100, UV lamp).

B. This culture was grown by the basic method as in A (above), without the inclusion of mitotic inhibitors. Note the greater density of nuclear staining seen in comparison with A (above) (LP x 100, UV lamp).

C. A high power photograph of B (above). Note the total absence of cytoplasmic staining (HP x 400, UV lamp).

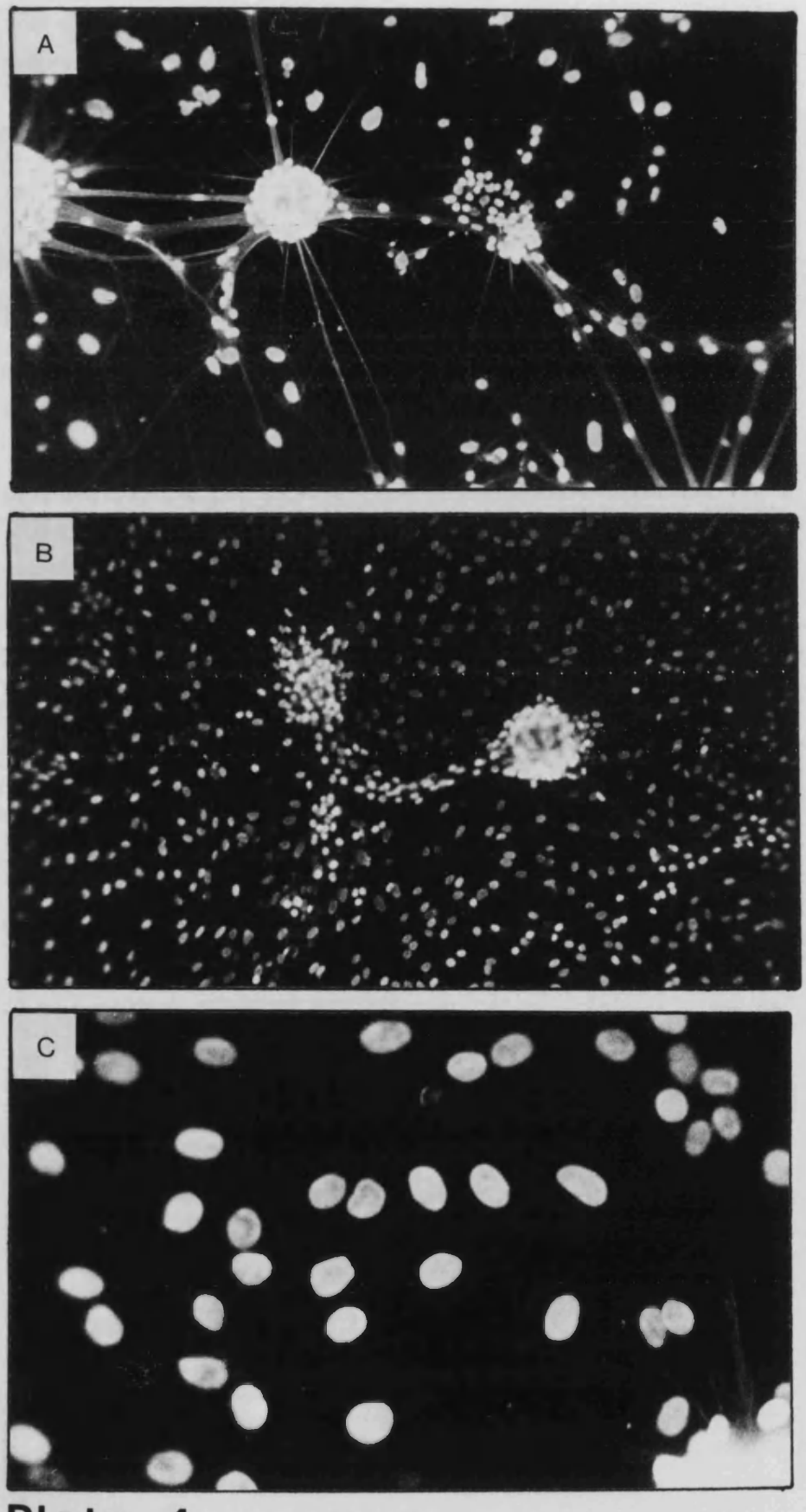


Plate 4

PLATE 5

A/B and C are rat spinal cord cell cultures (15-18 days) which have been grown by the basic method (Section 2.3.1.6), and fixed in 5% HAc/70% EtOH and processed by the indirect immunofluorescence method (Section 2.3.2.5(i)) using the 2F3 monoclonal antibody (see Table 10 p 201).

A. Note how (in comparison to the 1C3 monoclonal antibody, Plate 4) the staining appears to be limited to the nuclear envelope (LP x 100, UV lamp).

B. A high-power photograph of the central section of A (above).

C. This culture was processed as in A/B (above) with the sole omission of the 2F3 antibody. Two large neuronal clusters are just visible. This photograph serves as a universal control for the omission of primary antibody, and also in the case of non-fixation when an intra-cellular staining monoclonal antibody is tested (eg. 2F7, anti-neurofilament) (LP x 100, UV lamp).

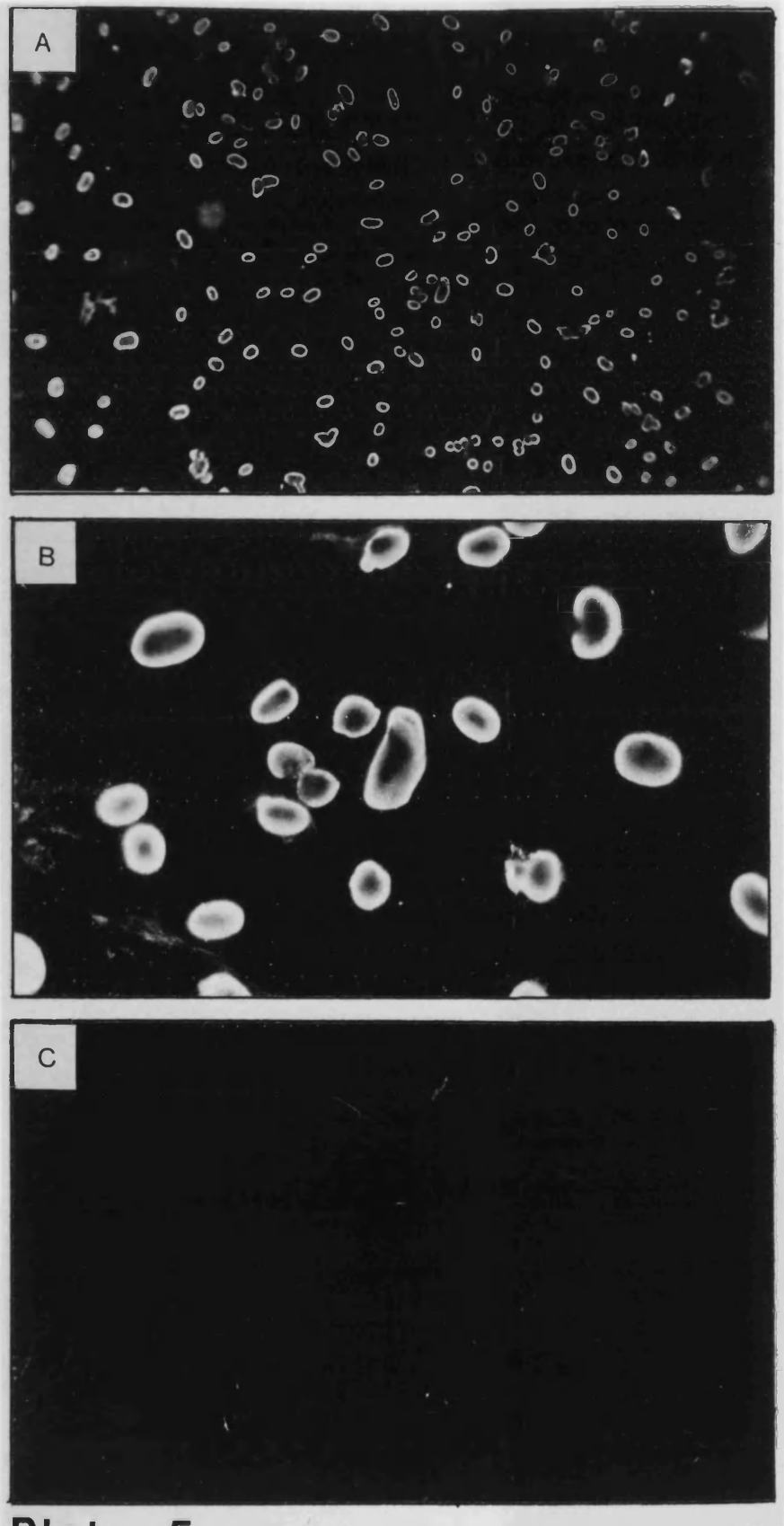


Plate 5

PLATE 6

A, B and c are all rat spinal cord cell cultures(15-18 days) grown by the basic method (Section 2.3.1.6) in SFM from day 3, fixed in 5%HAc/70% EtOH and processed by the indirect immunofluorescence method (Section 2.3.2.5(i)) using the 3G6 antibody (see Table10 p201).

A. Several cells with a bipolar morphology are strongly stained. Their long processes appear, in some cases, to trace the route between neuronal clusters (unstained) (LP x 100, UV lamp).

B. This photograph clearly shows the morphology and orientation of these bipolar cells. They appear to trace the route of neuronal processes from the cluster (just visible, right). Note the unstained nucleus. It seems most likely that they are Schwann cells (HP x 400, UV lamp).

C. This photograph is of a single example of the type of cell which stains with the 3G6 antibody (see B, above). The long processes transverse the surface of background cells whose nuclei (N) are clearly visible (HP x 400, Tunsten lamp).

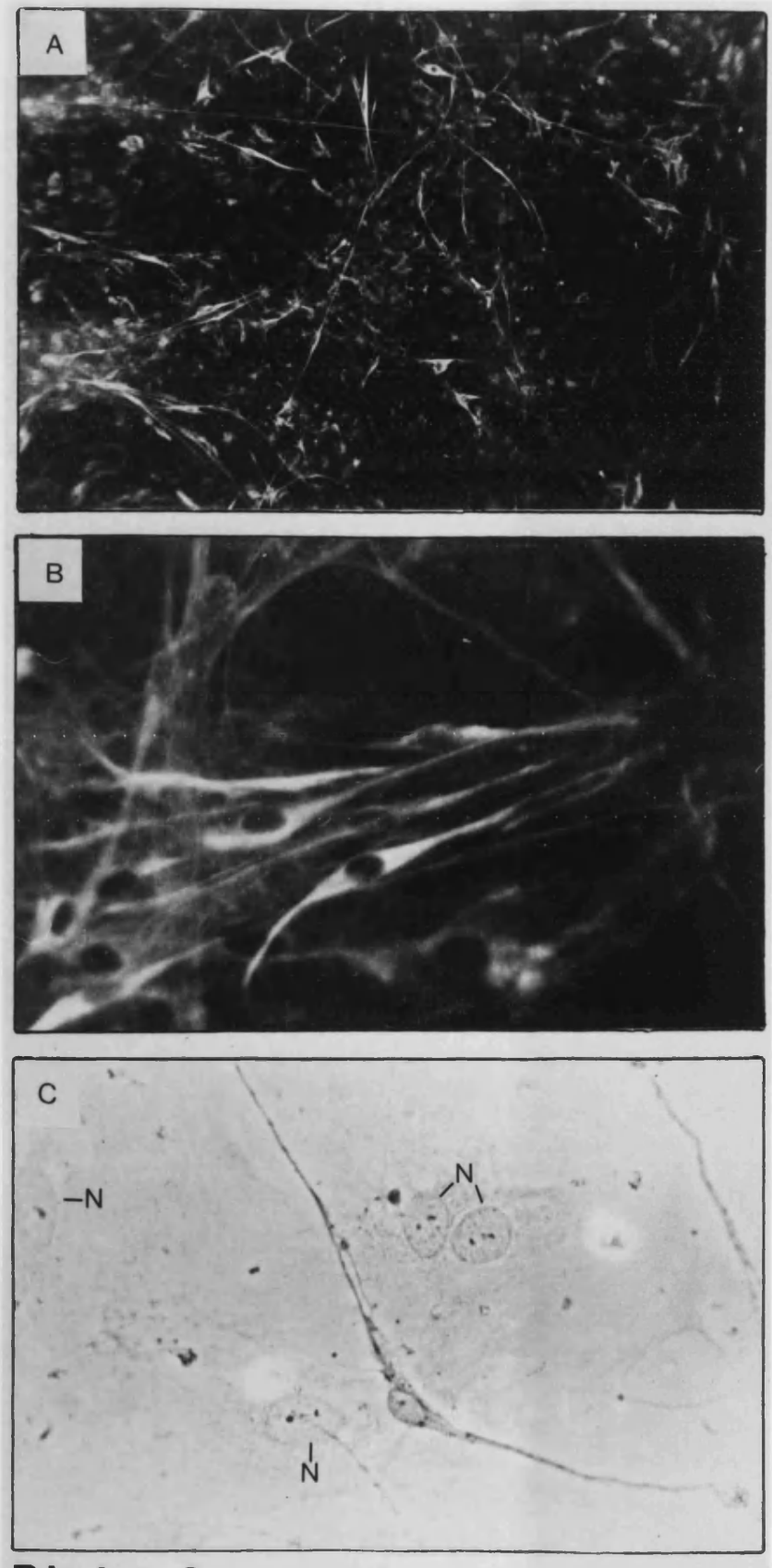


Plate 6

PLATE 7

A/B is a very young rat spinal cord cell cluster (day 2/3) grown from day 0 in SSM (Section 2.3.1.6). It has been processed, without prior fixation, by the indirect immunofluorescence technique using the 1B7 antibody (Table 10 p201).

A. A number of small, round cells have been indicated on the photograph. They occur within the neuronal cluster (HP x 400, Tungsten lamp).

B. The strongest surface-staining cells in this photograph are the ones numbered in A (above). For the sake of clarity, only number 6 has been individually identified (HP x 400, UV lamp).

C. This is an older rat spinal cord cell culture (day 15-18) grown and processed as in A/B (above). The staining is much weaker, and the small, round cells appear to develop an irregular shape and even send out fine processes. Their nature is unknown (HP x 400, UV lamp) (see Section 4.2.2(vi)).

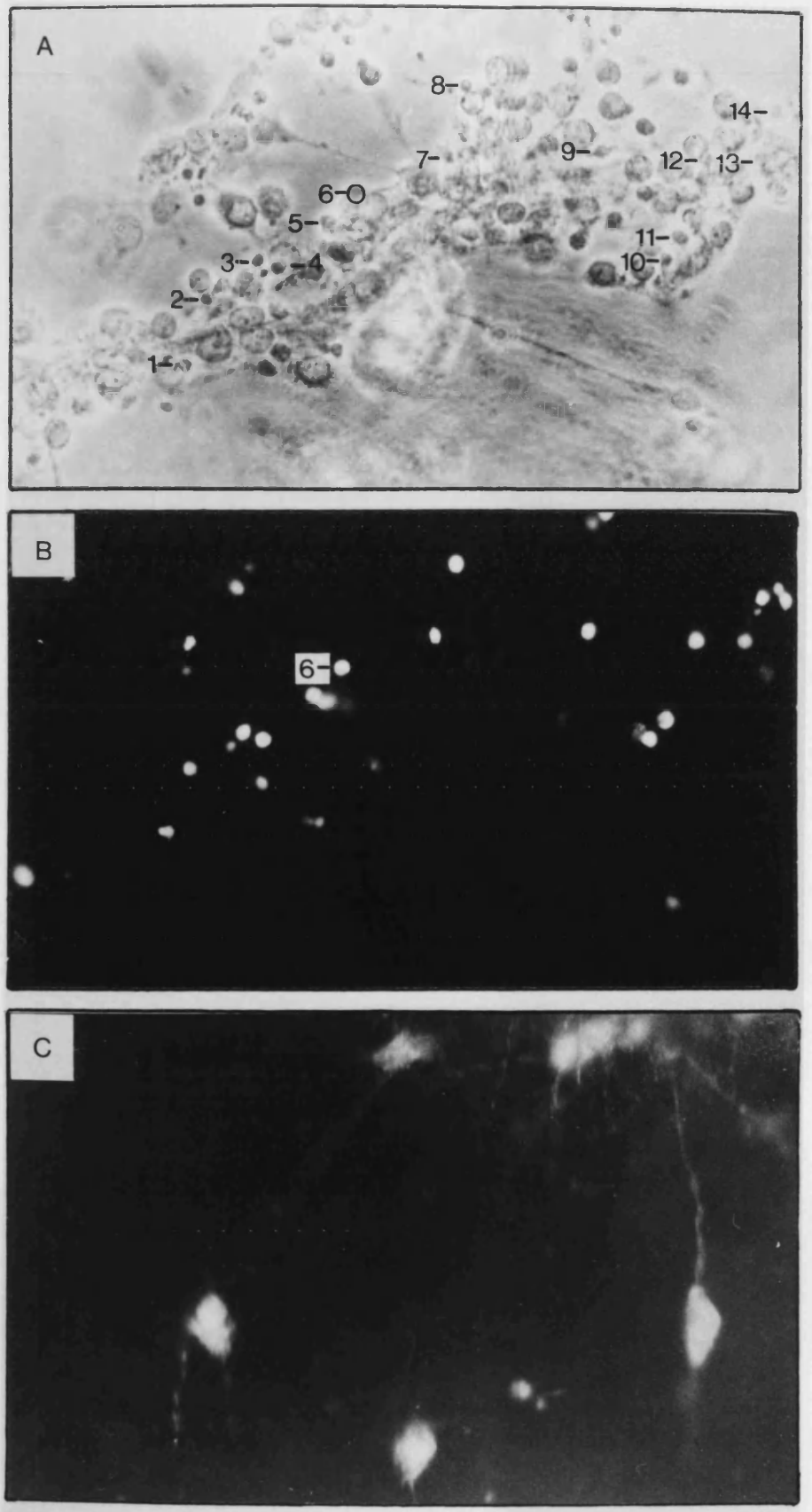


Plate 7

PLATE 8

A/B and C are both rat spinal cord cell cultures (day 12-15) which have been maintained in SFM containing a low serum concentration (2%) from day 3 (Section 3.1.3(iii)). They have been processed without prior fixation by the indirect immunofluorescence technique (Section 2.3.2.5(i)) using the 3C4a/b antibody (see Table 10 p201).

A. A single, small neuronal cluster (background cells are not in focus due to the 3-D nature of the cluster). (HP x 400, Tungsten lamp).

B. The surface staining produced by the antibody is particulate and appears to trace small individual tracts within a neuronal process (bottom centre) (HP x 400, UV lamp).

C. Another example of the staining produced by the 3C4a/b antibody. Note the neuronal specificity of the staining. Occasional small blobs of staining are active microglial cells which have ingested some of the labelled antibody complexes (LP x 100, UV lamp).

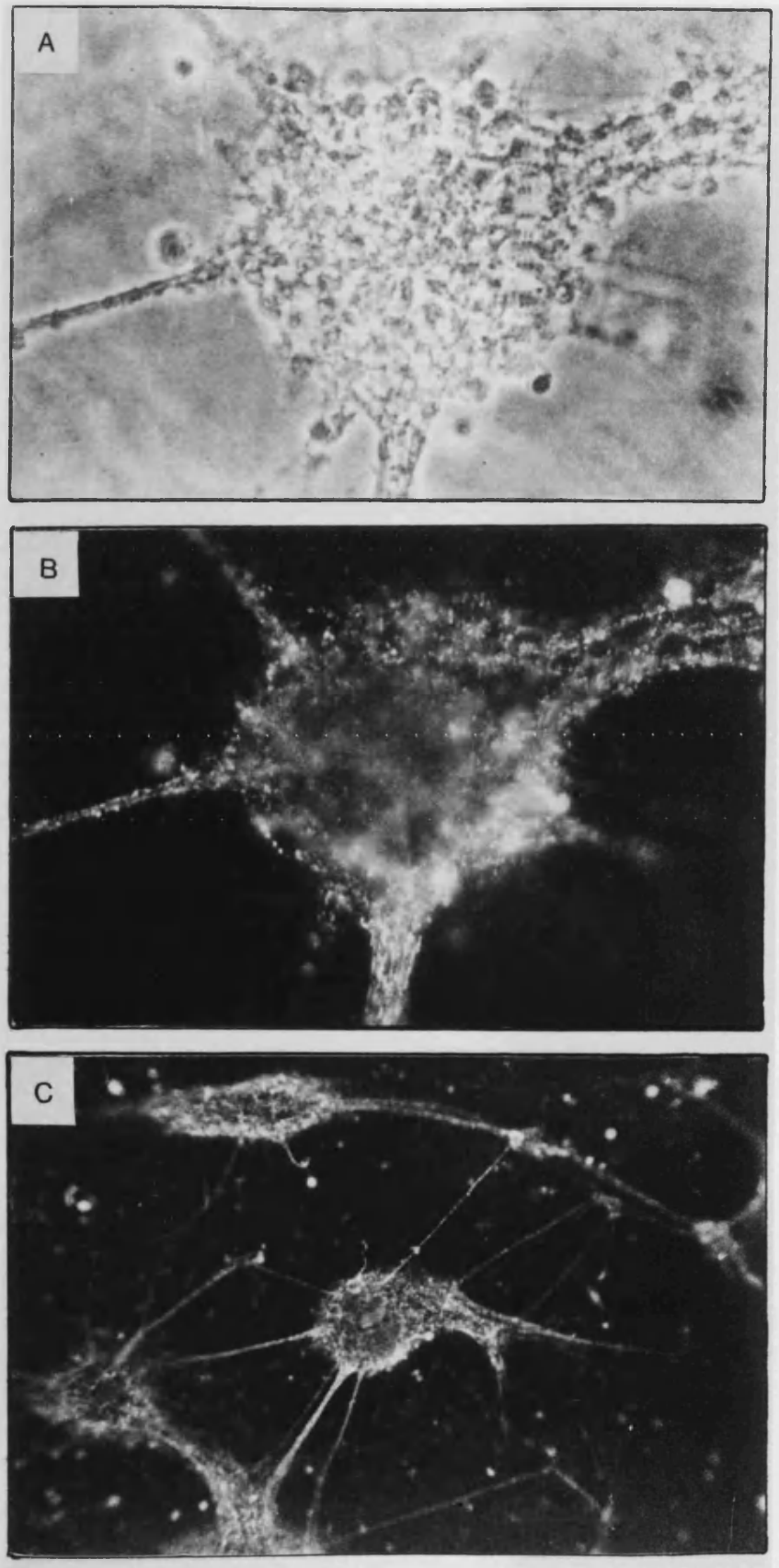


Plate 8

PLATE 9

A, B and C are examples of the three different types of staining which have been seen when the 3C4a/b antibody has been tested on rat spinal cord cell cultures, fixed in 5%HAc/70% EtOH and processed by the indirect immunofluorescence technique (2.3.2.5(i)).

A. Type A staining is more intense, and less particulate than seen with live cultures (see Plate 8(C)). the staining is still specific to the clusters and processes with absent or only weak background staining (LP x 100, UV lamp).

B. Type B staining is characterised by strong staining of neuronal clusters and processes, plus background cells (LP x 100, UV lamp).

C. Type C staining is characterised by strong staining of background cells only. The neuronal processes are completely unstained and produce a strange "negative-like" effect as they run over the surface of the background cells (LP x 100, UV lamp) (see Section 4.2.2(vii)).

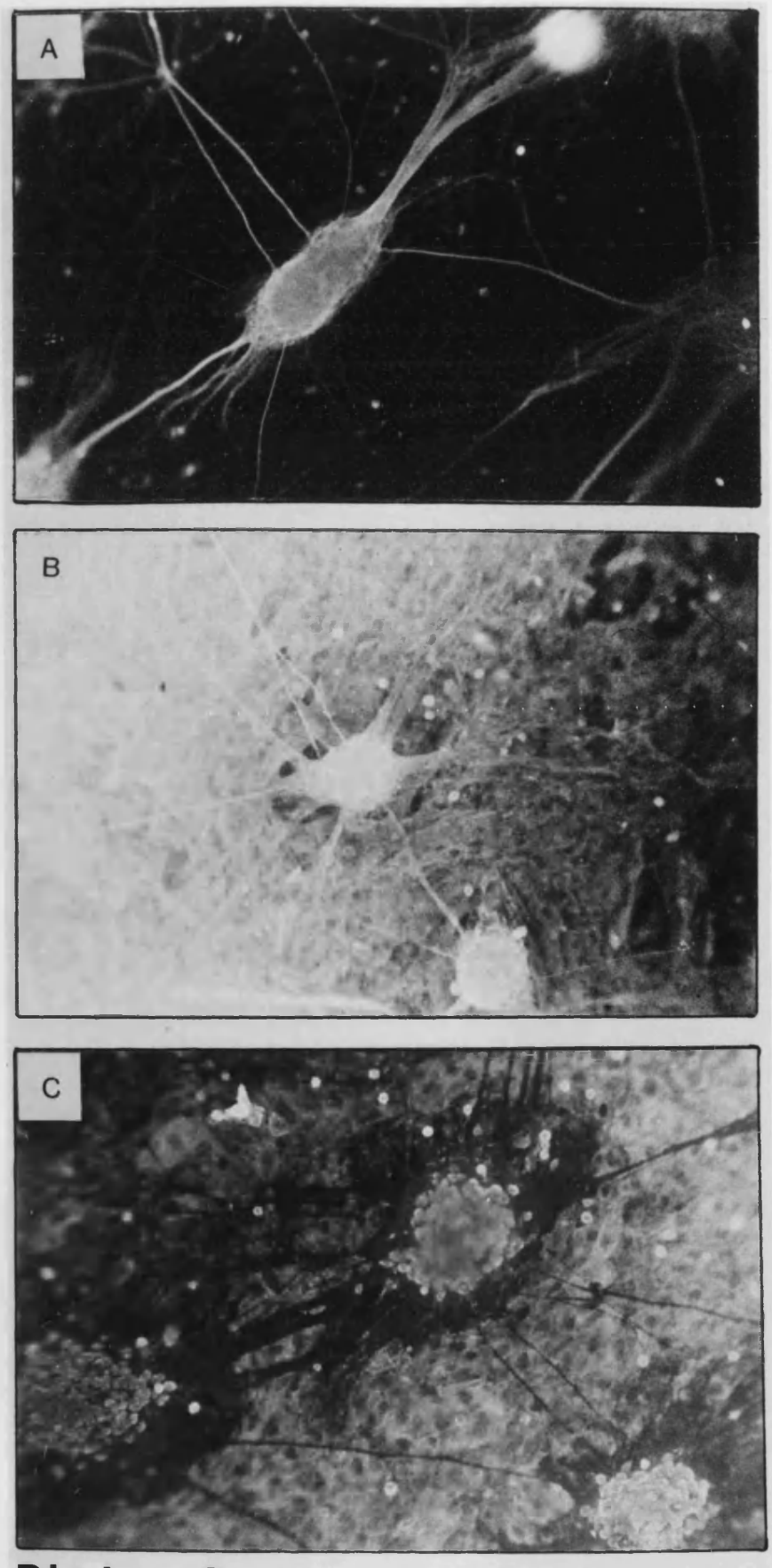


Plate 9

PLATE 10

A. This photograph is a high-power shot of the Type C staining previously described with the 3C4a/b antibody (Section 3.2.5 and Plate 9(C)) (HP x 400, UV lamp).

B/C. These rat spinal cord cell cultures have been fixed by the alternative acetone method (Section 3.2.7) and then processed for indirect immunofluorescence by the usual technique (Section 2.3.2.5(i)), using the 3C4a/b antibody. The staining becomes clearer and more intense and, where individual background cells can be isolated, two different types of staining can be identified.

B. The staining is intense around the nucleus and becomes more diffuse towards the cell periphery, defining a "lace-like" morphology (HP x 400, UV lamp).

C. The staining is localised to a fibrous network within the cell cytoplasm (HP x 400, UV lamp).

It is possible that the cell seen in B is a fibroblast, while the cell seen in C is a Type I astrocyte. These cells appear identical under tungsten lamp illumination.

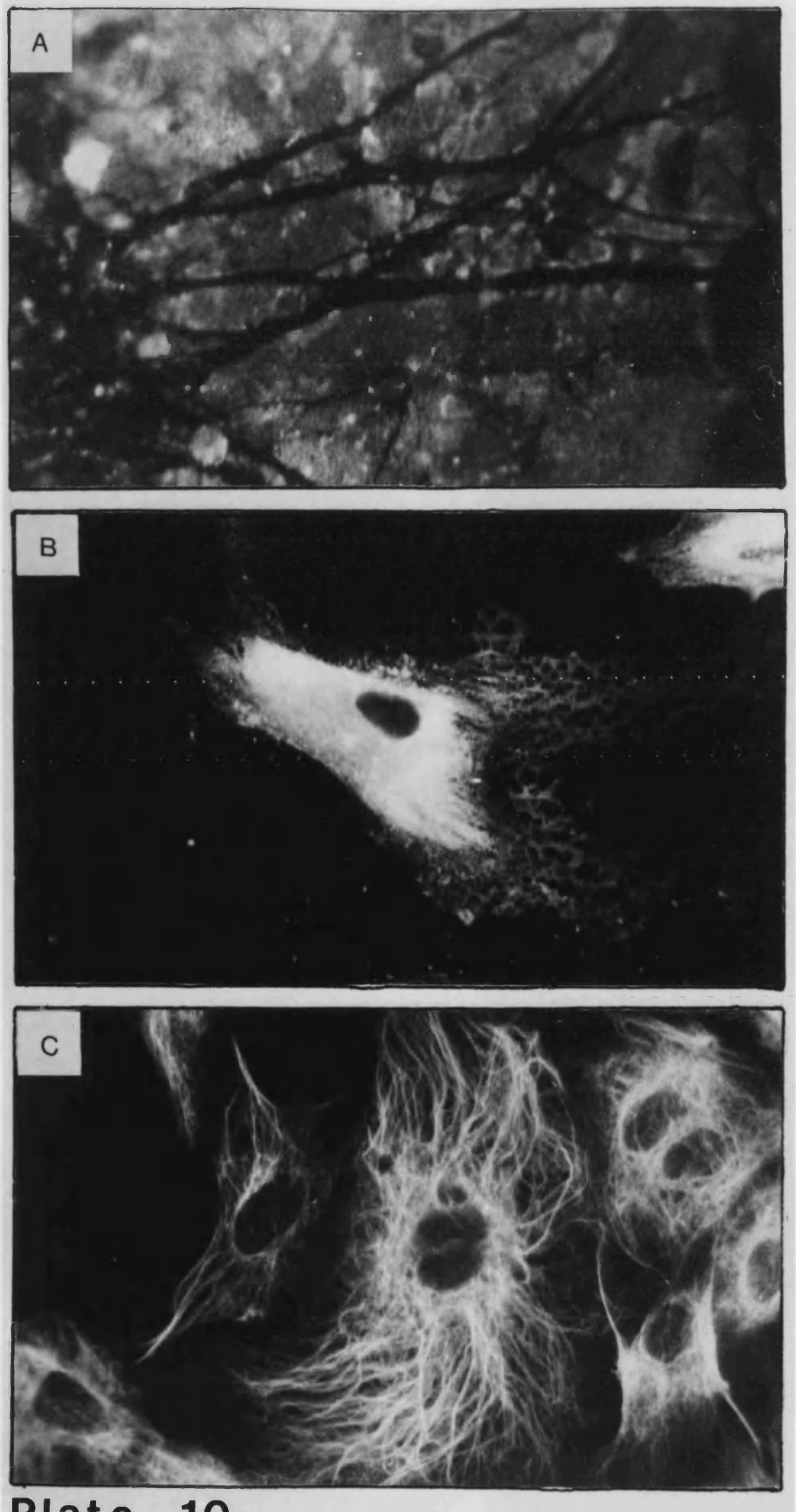


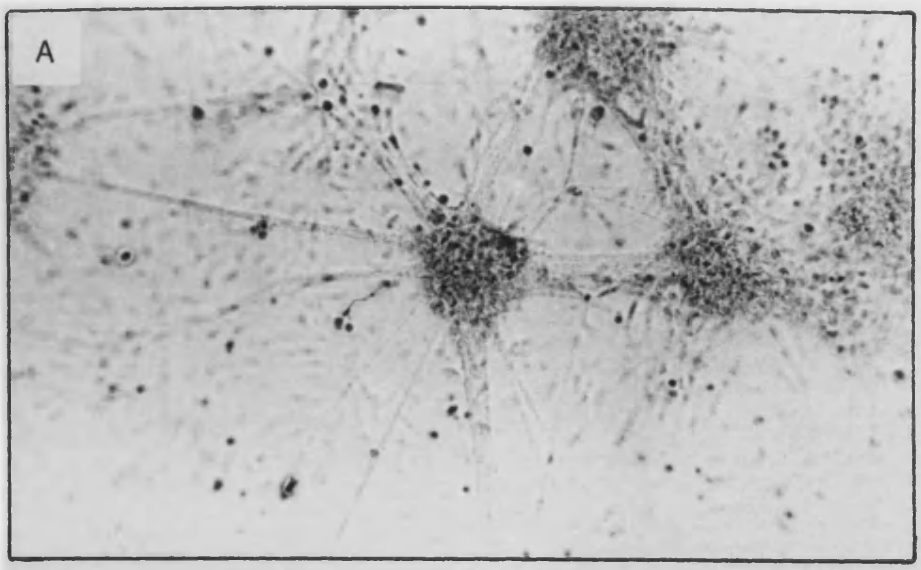
Plate 10

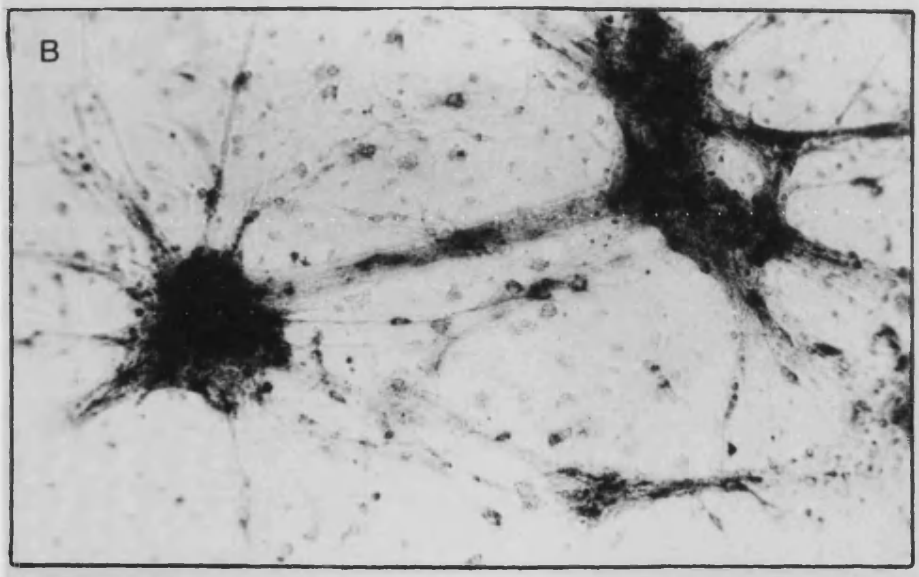
PLATE 11

A. Foetal rat spinal cord cell culture (day 12-15, grown in SFM from day 3). This live culture was processed by the localisation of acetylcholinesterase technique with the inclusion of the inhibitor neostigmine bromide (Sections 2.3.1.12 and 3.1.8). It serves as a negative control for B and C (below) (LP x 100, Tungsten lamp).

B. A culture from the same preparation, and grown and processed as in A (above) , but with the exclusion of the acetylcholinesterase inhibitor. Dark brown staining is seen concentrated in the neuronal clusters (LP x 100, Tungsten lamp).

C. Another example of the above staining procedure in a different (18 day) rat culture. The intense staining is concentrated in the neuronal clusters (LP x 100, Tungsten lamp).





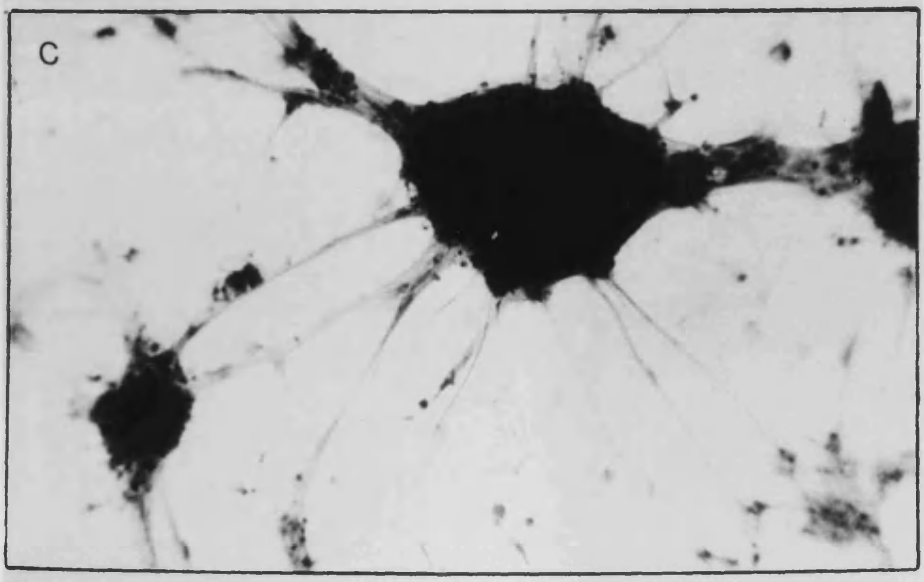


Plate 11

PLATE 12

A, B and C are all 15-18 day rat spinal cord cell cultures grown by the standard method (Section 2.3.1.6) in SFM from day 3. They have all been fixed in 5%HAc/70% EtOH and processed by the indirect immunofluorescence method (Section 2.3.2.5(i)) using human serum (1:10 dilution) as the primary antibody source and goat anti-human IgG/IgM-FITC conjugate to detect antibody binding (LP x 100, UV lamp).

A. TYPE I staining pattern - primarily of neuronal clusters and processes (most common).

B. TYPE II staining pattern - astrocytes are primarily stained (rare).

C. TYPE III staining pattern - cell nuclei are primarily stained (rare).

The staining patterns above were seen in the same frequency and at (subjectively) the same intensity, whether the serum was from a normal (control) individual, or from a patient suffering from Motor Neurone Disease. (see also Section 3.1.10(i) and 4.1.6 (i)).

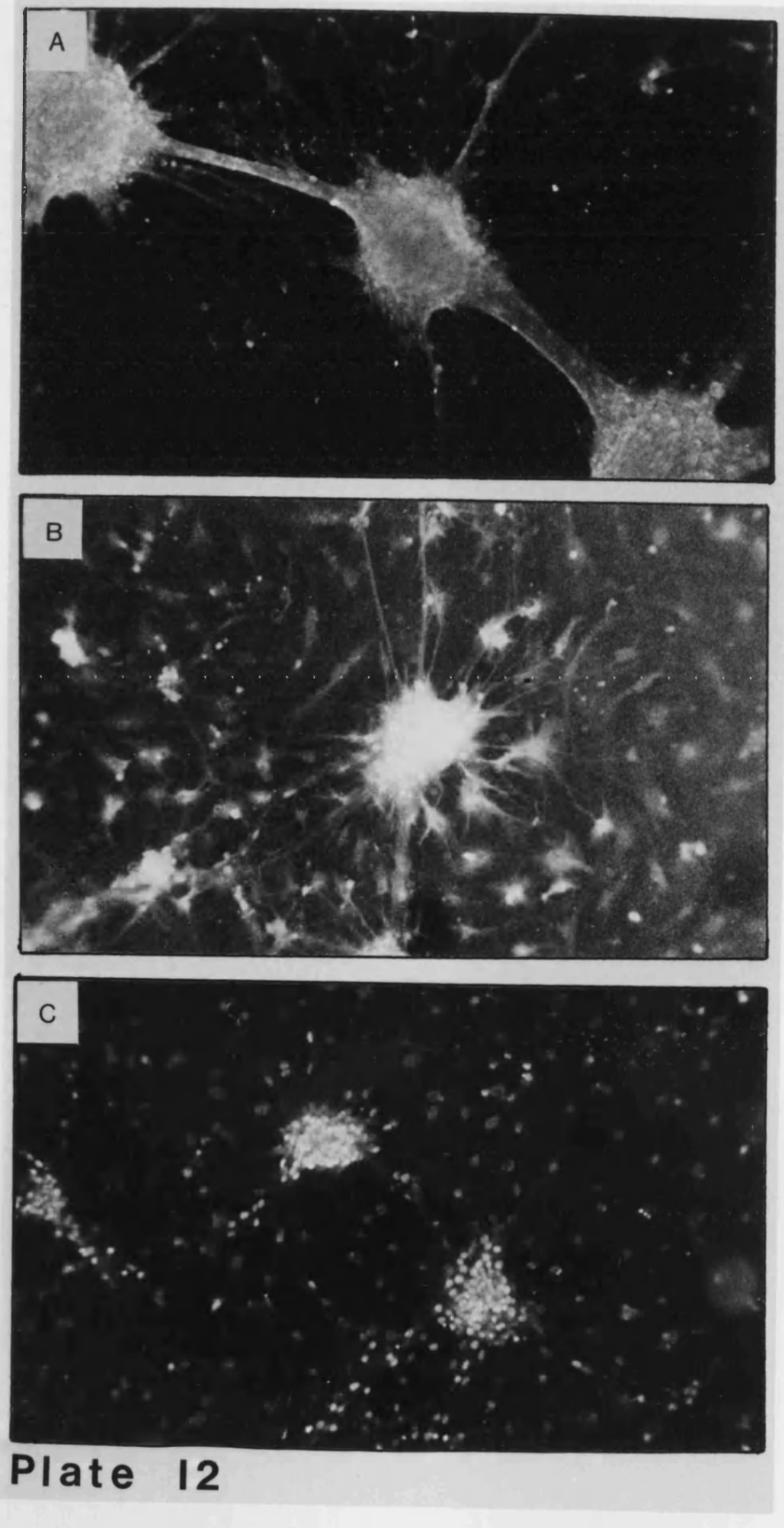


Plate 12

PLATE 13

A/B and C are both rat spinal cord cell cultures (day 15-18) grown by the standard method (Section 2.3.1.6) in SFM from day 3.

A/B. This culture has been processed live by the indirect immunofluorescence method (Section 2.3.2.5(i)) using normal human serum (neat) as the primary antibody source and goat anti-human IgG/IgM-FITC conjugate to detect antibody binding.
(A:LP x 100, Tungsten lamp; B:LP x 100, UV lamp).

C. This culture has been processed as in A/B (above) but using serum from a patient with MND (LP x 100, UV lamp).

The results are typical-surface antibody binding was seen in all cases and was (subjectively) of equal intensity. The staining was largely specific for the neuronal clusters and processes.
(see also Sections 3.1.10(i) and 4.1.6(i)).

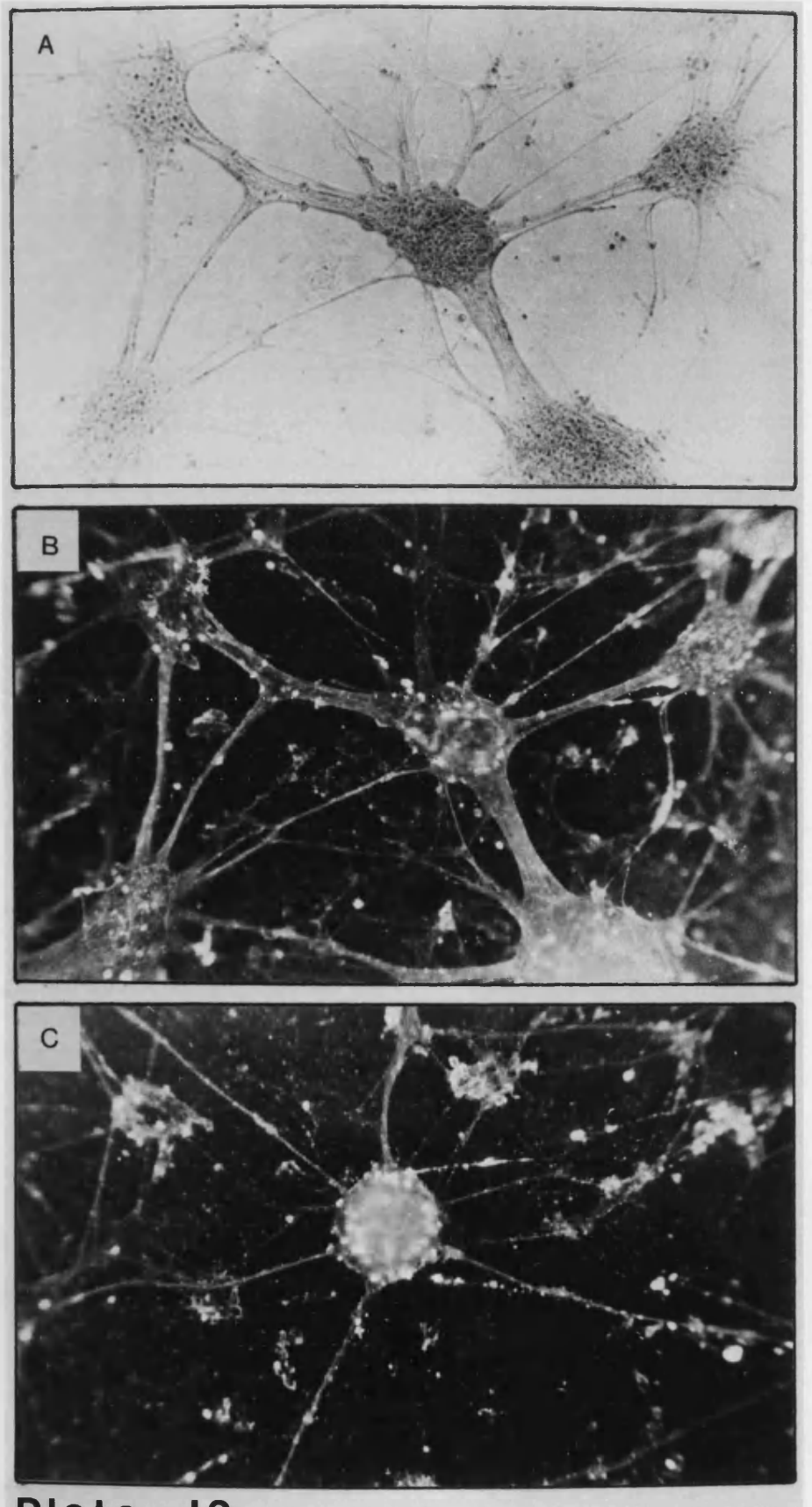


Plate 13

PLATE 14

A. Rat spinal cord cell culture (day 15-18) grown by the basic method (Section 2.3.1.6) in SFM from day 3. Neuronal clusters and processes are the most obvious morphological feature at this magnification (LP x 100, Tungsten lamp).

B. A separate culture produced from the same cell preparation as used for A (above), but grown with the additional inclusion of foetal human skeletal muscle extract (200µg/ml) in the medium from day 0. The culture was photographed on the same day as A (above) and at the same magnification (LP x 100, Tungsten lamp). The neuronal clusters are typically larger, and the processes thicker and more numerous.

C. Example of a dead or dying culture (day 21+). Cultures which have suffered a toxic stimuli also appear thus. The neuronal clusters become phase-dark and the processes tend to disappear (LP x 100, Tungsten lamp).

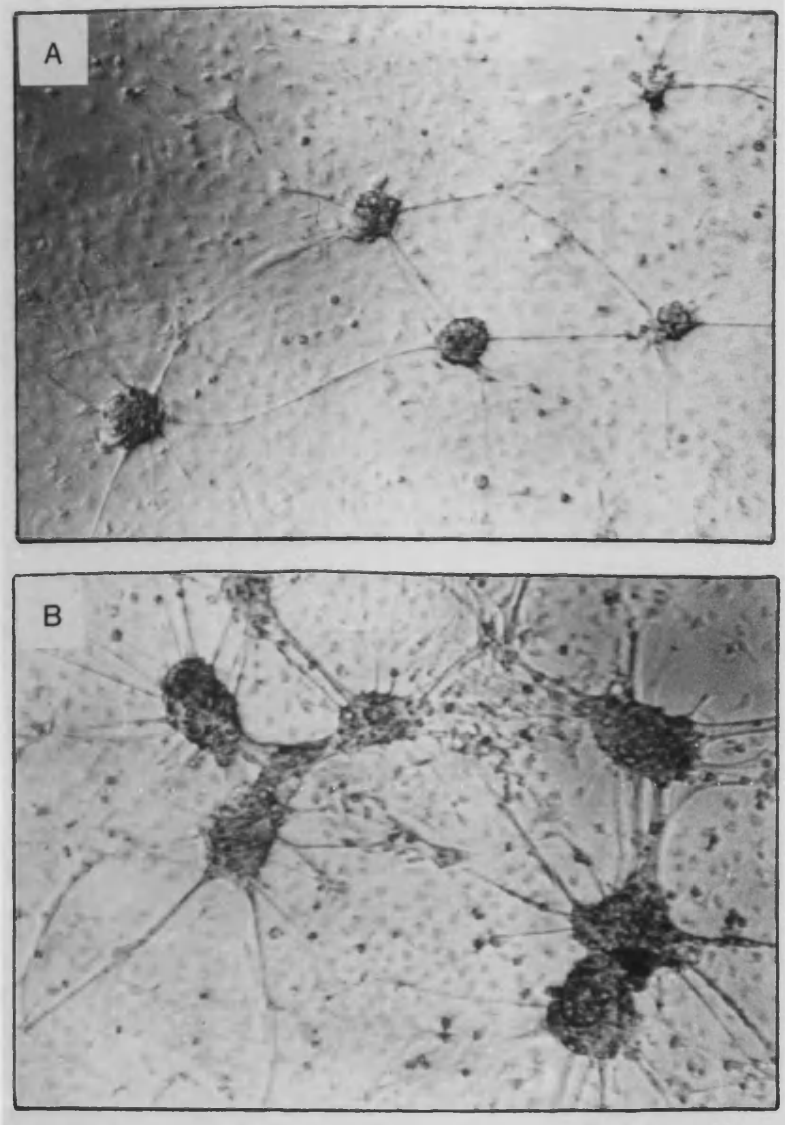


Plate 14

PLATE 15

A. Example of a very large rat spinal neurone cluster produced by the inclusion of foetal calf skeletal muscle extract (200µg/ml) in the basic SFM. The culture (day 21) was stained for neurofilament protein using the 2F7 monoclonal antibody (see Table 10 p201)(LP x 100, UV lamp).

B. Example of a large rat spinal neurone cluster produced by the inclusion of adult human skeletal muscle extract (200µg/ml) in the basic SFM. The culture (day 21) was stained as in A (above) (LP x 100, UV lamp).

C. Example of rat spinal neurone cluster (from the same preparation as A and B) grown in SFM alone (from day 3). The culture (day 21) was stained as in A and B (above) (LP x 100, UV lamp). Note the gradual disappearance of the neuronal processes at this age.

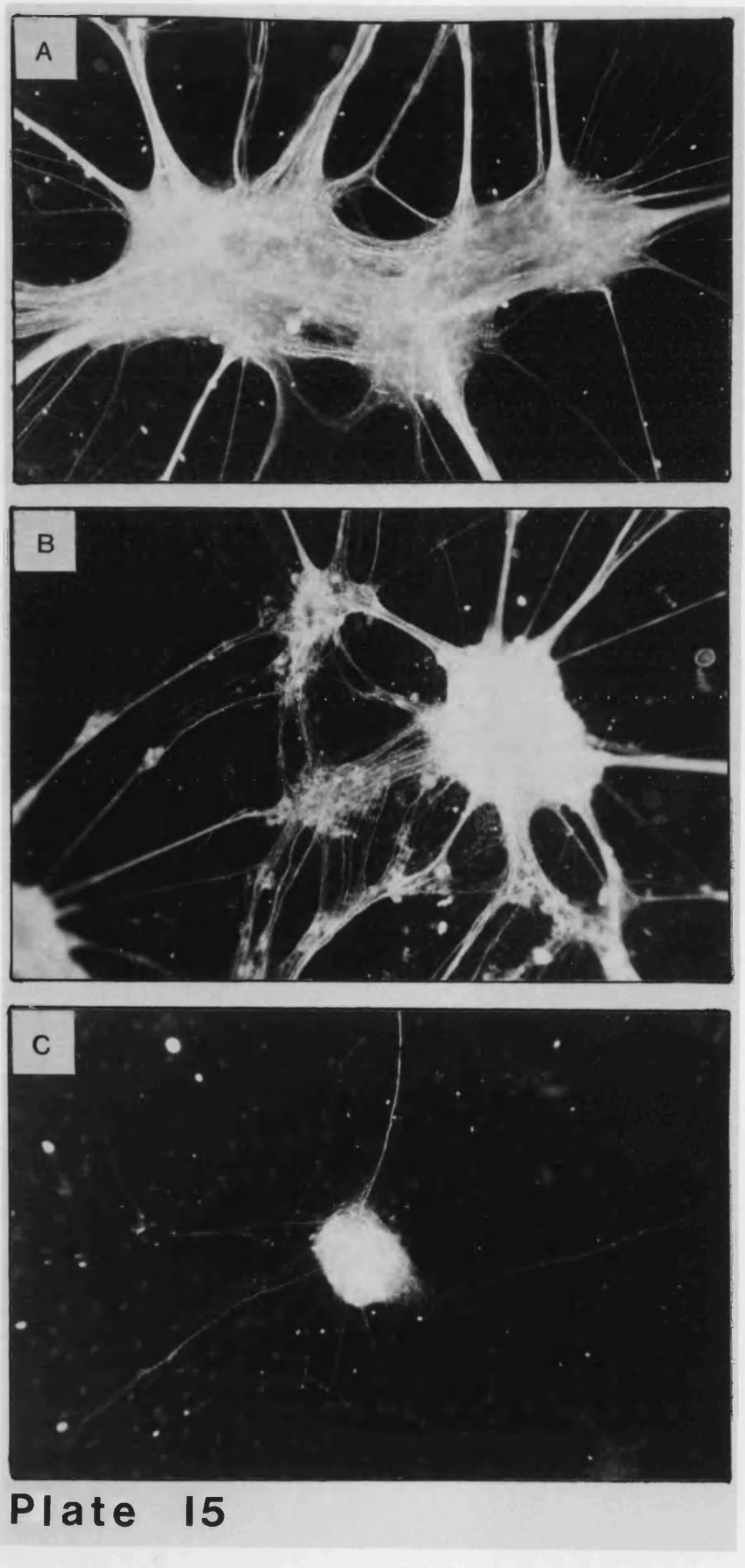


Plate 15

PLATE 16

A. Human foetal spinal neurone cluster (day 15-18) grown throughout in HM-A. Note the large number of background cells (nuclei visible) which mainly obscure the neuronal processes. The culture was stained with Ehrlichs haematoxylin /eosin (HP x 400, Tungsten lamp).

B. Human foetal spinal neurone cluster (day 15-18) grown from day 6-9 in SFM. The neuronal cell bodies and processes are more distinct than in A (above). Only a few background cells are visible. The extent of the cytoplasm of a single fibroblast-like cell is indicated with arrows. The single, large, oval nucleus is clearly visible (N).

It is interesting to note that an area of the culture (bottom right) appears totally devoid of any background cells. No neuronal processes are seen to transverse this area. This is consistent with the substrate-mediated theories of growth cone advancement (Section 1.1.5). The culture was stained as in A (above) (HP x 400, Tungsten lamp).

C. Human foetal spinal cord cell culture, processed for the localisation of acetylcholinesterase activity (Sections 2.3.1.12 and 3.1.8). The dense brown staining is concentrated in the neuronal clusters (LP x 100, Tungsten lamp).

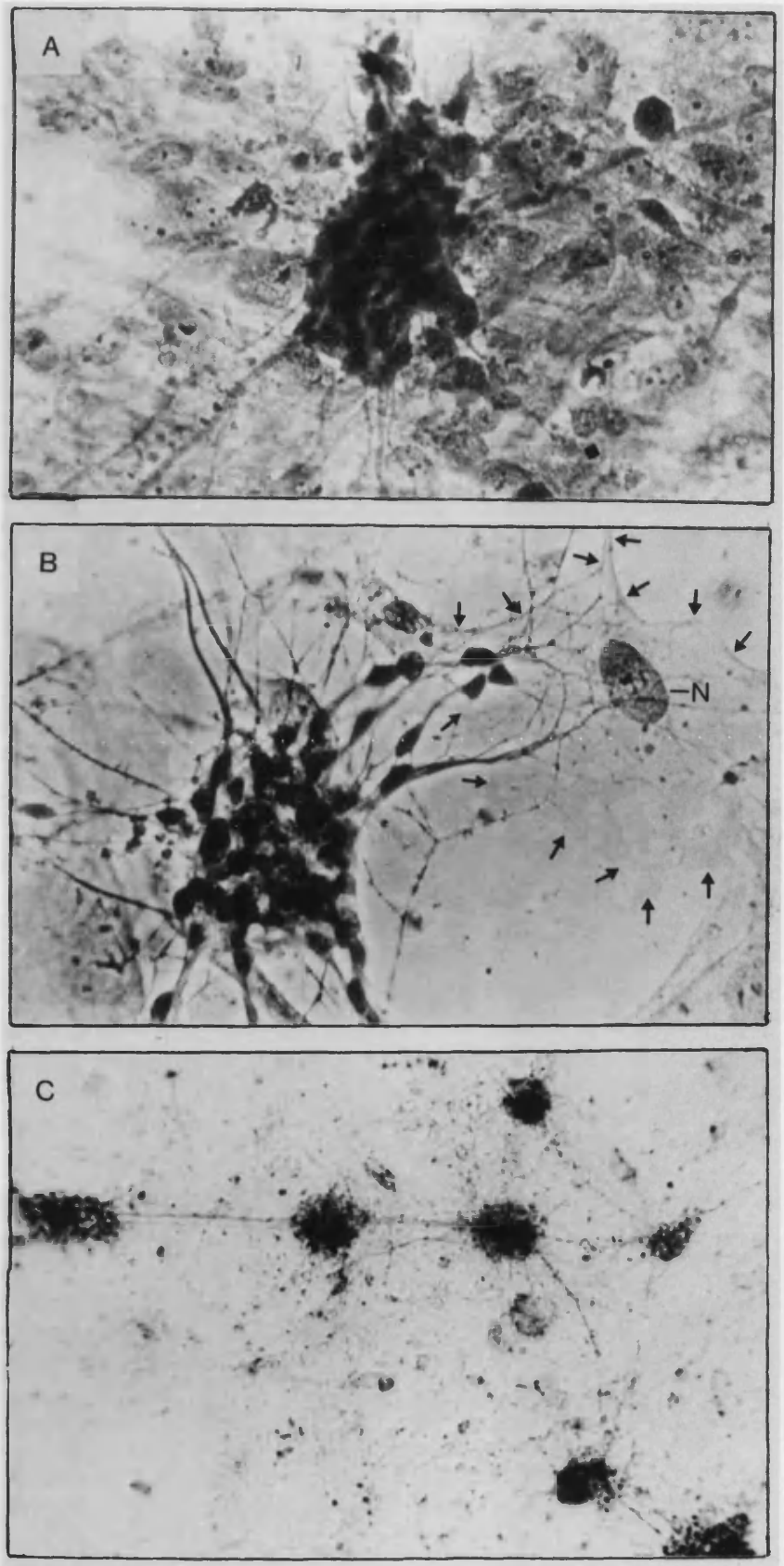


Plate 16

PLATE 17

A. Human foetal spinal neurone clusters (grown in SFM from day 6-9, fixed in 5% HAc/70% EtOH day 18). The culture was stained for neurofilament protein with the 2F7 monoclonal antibody (see Table 10 p201). This demonstrates cross-reactivity with human neurofilament protein. Note weak staining of background cell nuclei as mentioned elsewhere (Section 4.2.2(i))(LP x 100, UV lamp).

B. Human foetal spinal neurone cluster (grown as in A above). The culture was processed live on day 15 for the localisation of tetanus toxin binding (Section 2.3.1.13 and 3.1.9). Note specificity of staining confirming (with A, above) the neuronal identity of the clusters and processes (HP x 400, UV lamp).

C. Human foetal spinal cord cell culture (grown as in A and B above, fixed in 5% HAc/70% EtOH). This photograph demonstrates the cross-reactivity of the 1C3 monoclonal antibody (see Table 10 p 201) for human cell nuclei (HP x 400, UV lamp).

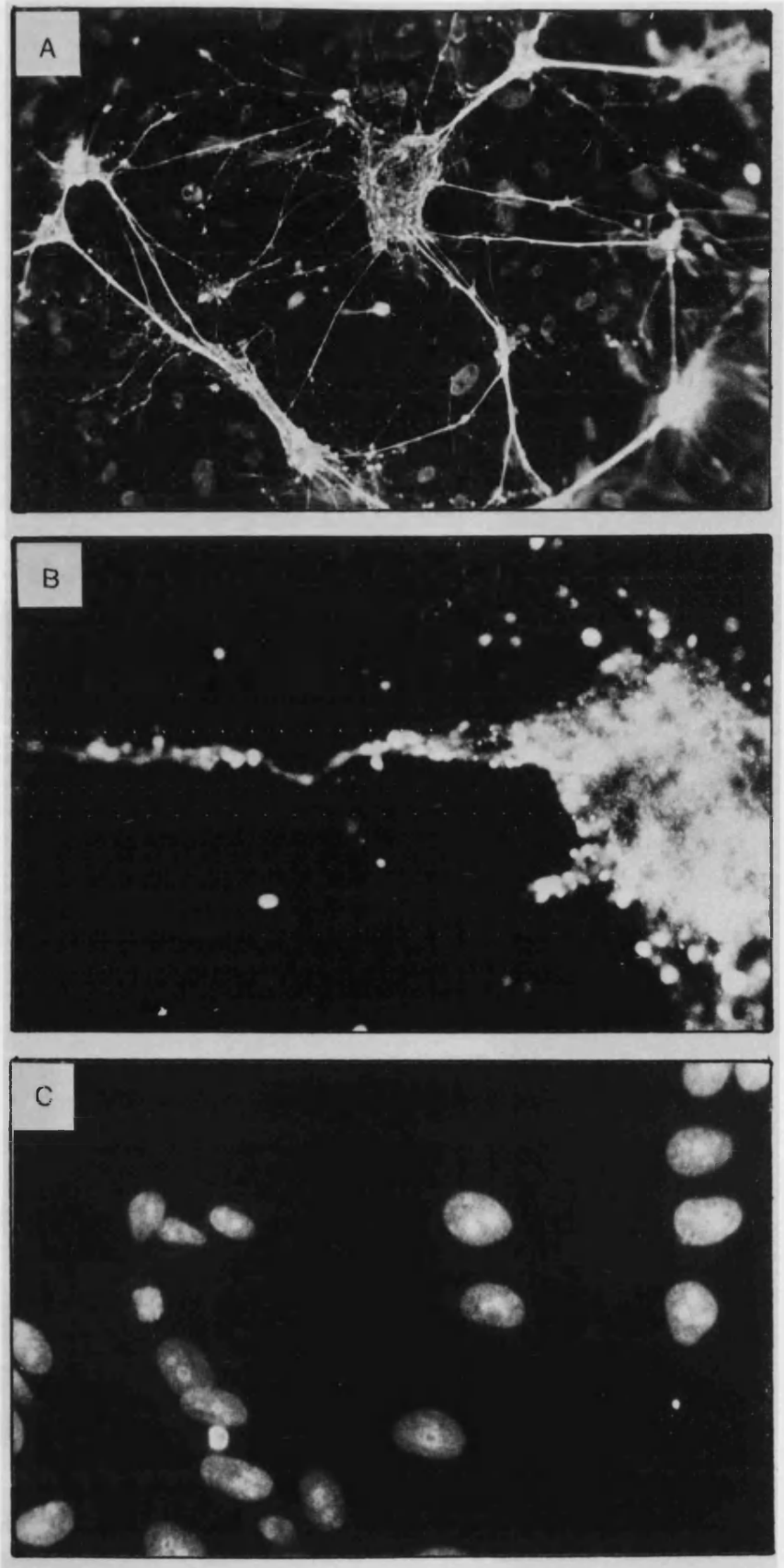


Plate 17

PLATE 18

A. Very young (3/4 days) human foetal spinal neurone culture (grown in HM-A; live cells). The clusters are small, and the processes poorly formed (LP x 100, Tungsten lamp).

B. The live culture in A (above) had been processed by the immunofluorescence technique (Section 2.3.2.5(i)) using the 3C4a/b antibody (see Table 10 p201). Strong surface staining is seen, specific to the neuronal clusters and processes demonstrating cross-reactivity with human neurones (LP x 100, UV lamp).

C. Another field of view of the same culture as in A and B (above). (LP x 100, UV lamp).

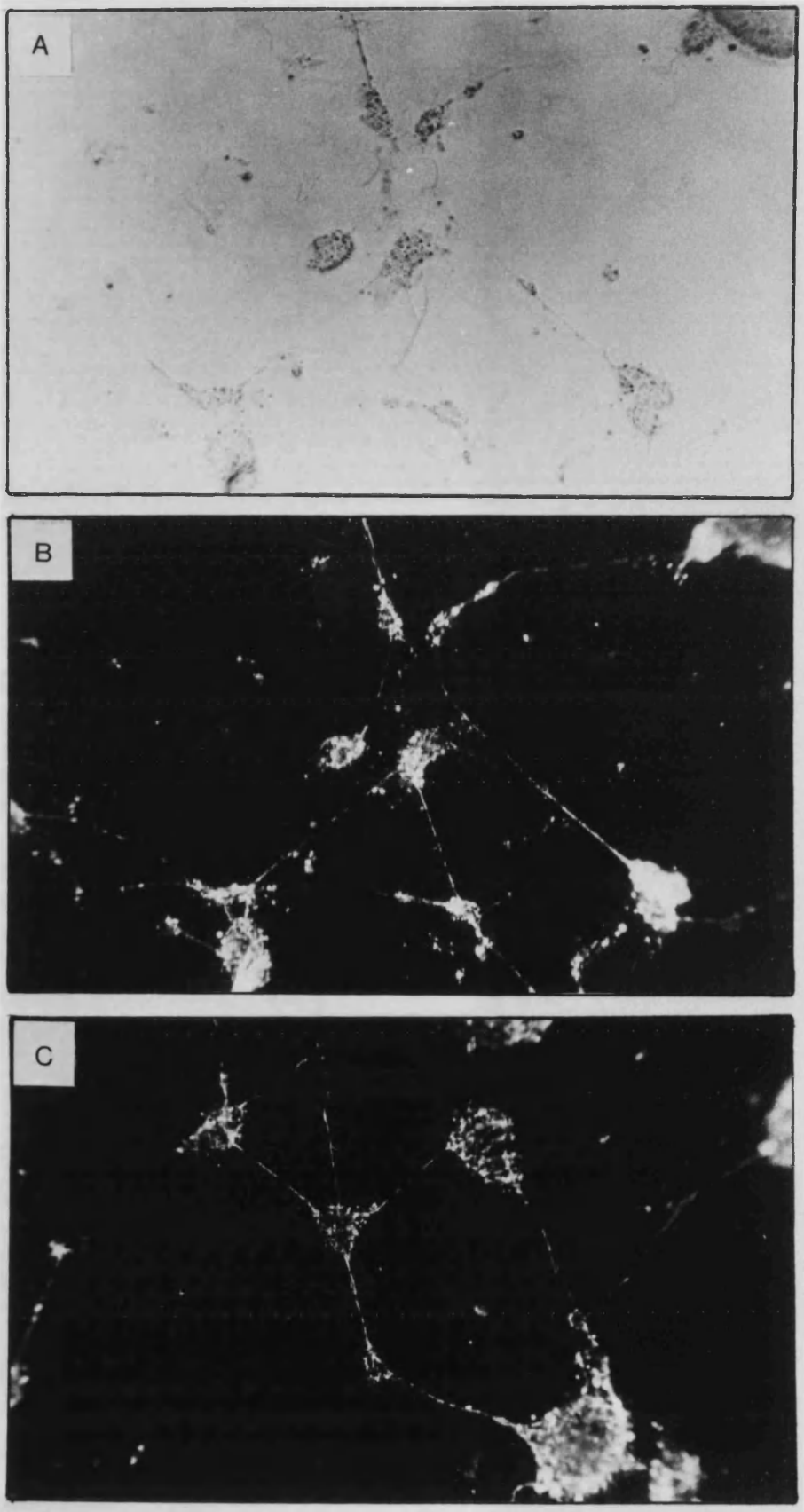


Plate 18

PLATE 19

A. Human foetal spinal cord cell culture (day 21; grown in HM-A throughout; live cells). The non-neuronal cells have continued to multiply and now practically obscure the neuronal clusters and processes (LP x 100, Tungsten lamp).

B. The live culture in A (above) had been processed by the immunofluorescence technique (Section 2.3.2.5(i)) using the 3C4a/b antibody (see Table10 p201). Neuronal specific surface staining is still strong enough for photography (this is not the case in cultures grown in SFM from day 6-9).

C. Another field of view of the same culture as in A and B (above)(LP x 100, UV lamp).

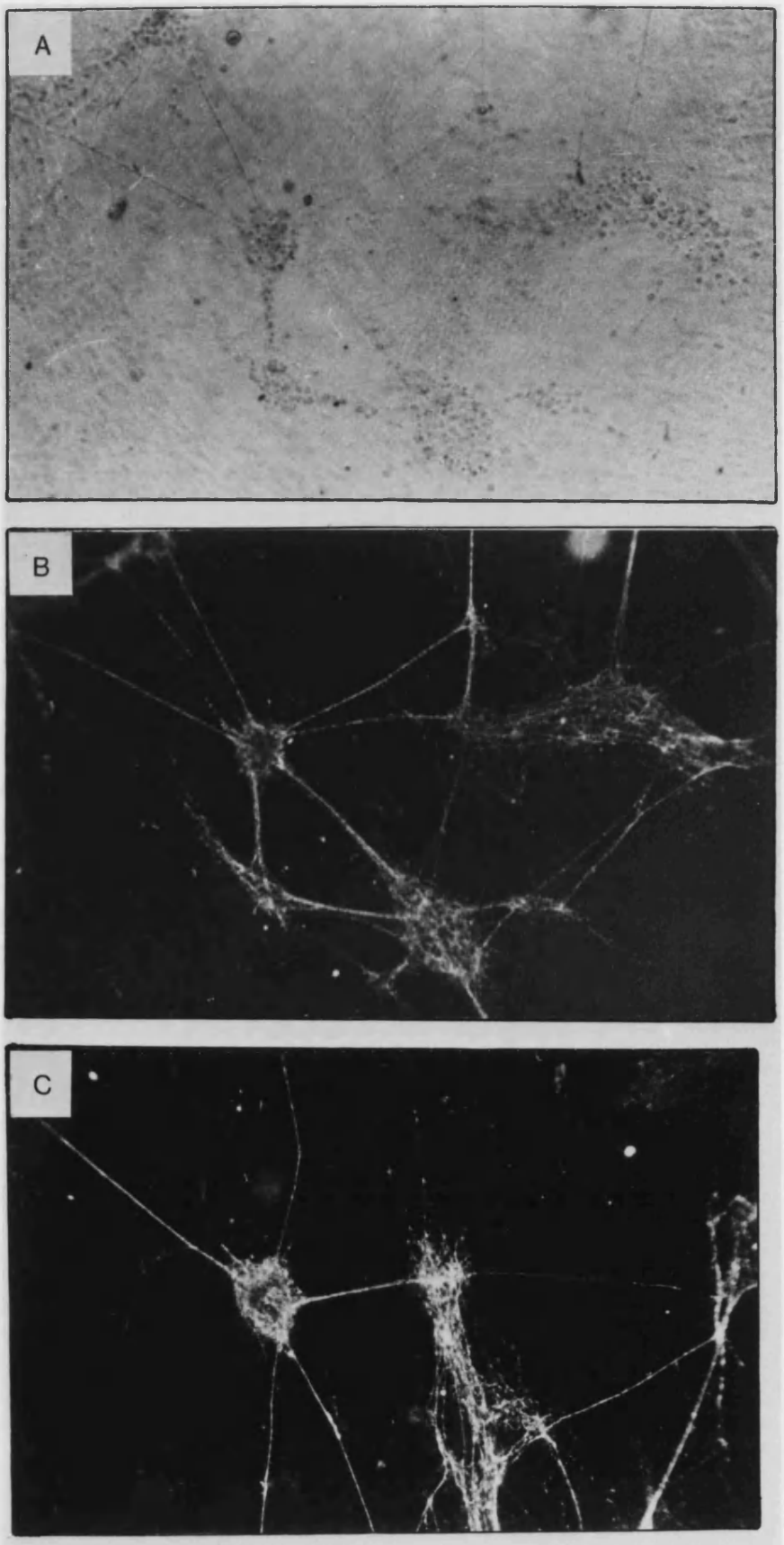


Plate 19

PLATE 20

A, B, C.

Three different human spinal cord cell/skeletal muscle co-cultures (grown from day 6-9 in SFM: A = live cells, B and C are fixed in 5%HAc/70% EtOH and lightly stained with Ehrlichs haematoxylin/eosin). Neuronal clusters are seen in close proximity to well developed myotubes. Fine neuronal processes are seen to connect the clusters and also in apparent innervation of the myotubes (LP x 100, Tungsten lamp).

NC = neuronal cluster

M = myotube

NP = neuronal process.

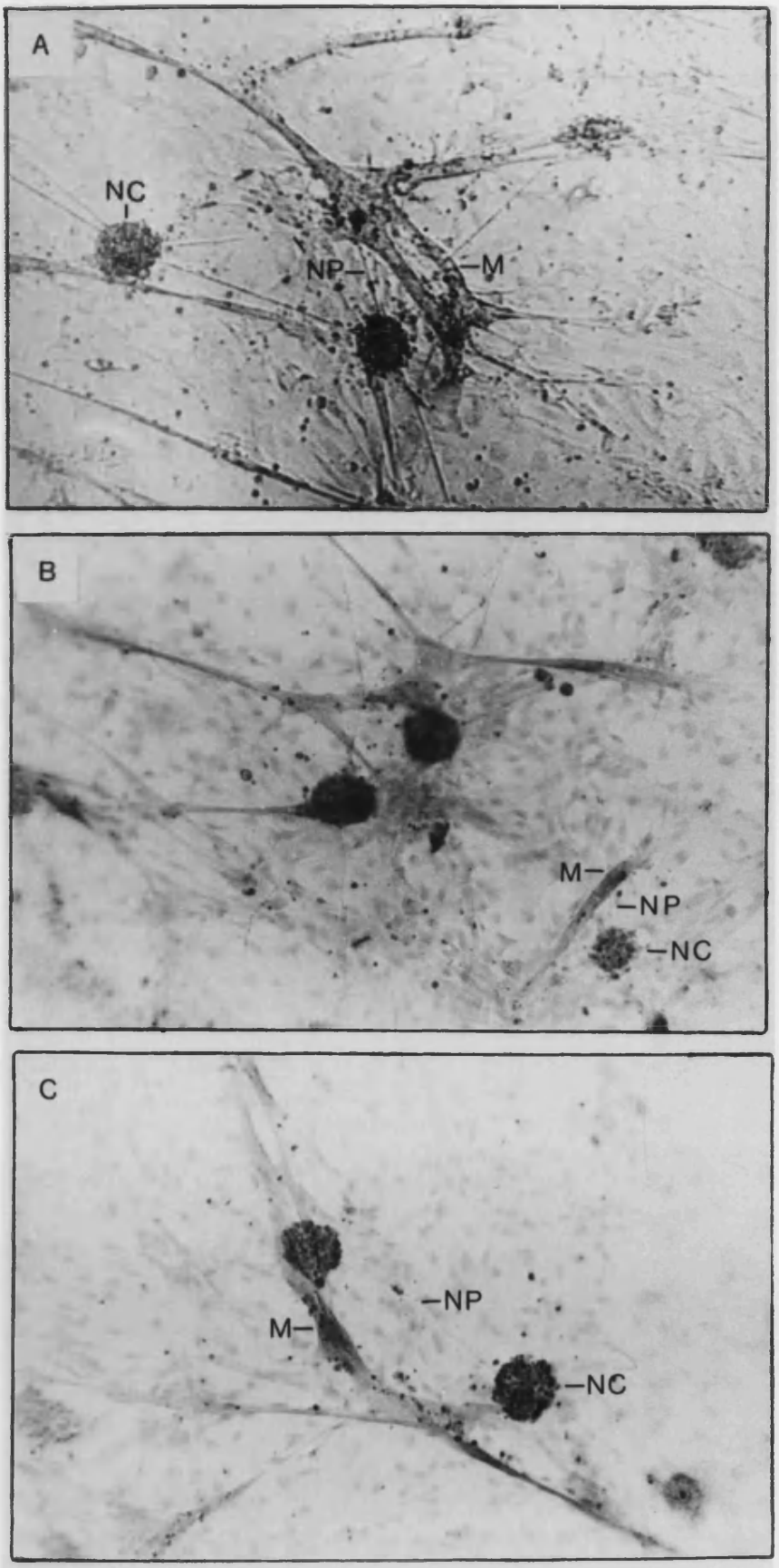


Plate 20

PLATE 21

A. These are immunoglobulin secreting hybridoma cells which have been fixed in acetone and processed by the indirect immunofluorescence technique (Section 2.3.2.5(i)). Practically every cell is stained (LP x 100, UV lamp; Tungsten equivalent not shown).

B/C. These are the same cells as above which have been processed as above without prior fixation. Many cells are only weakly stained and a considerable number are unstained (indicated with arrows). Only the highly vacuolated, secreting cells appear to be strongly stained (See Section 3.2.6).
(B = LP x 100, Tungsten lamp; C = HP x 100, UV lamp).

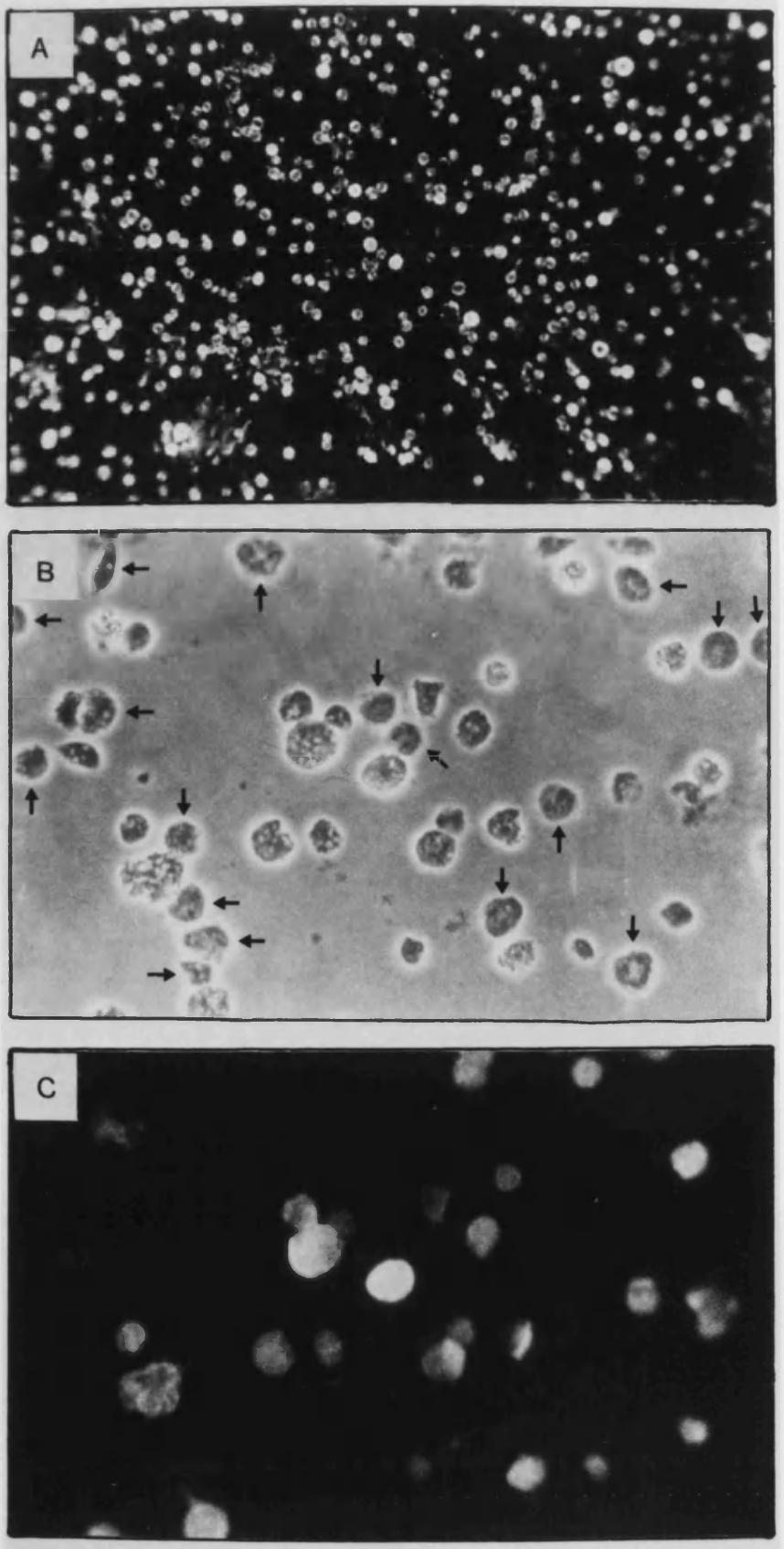


Plate 21

PLATE 22

This plate is of an SDS-PAGE/Western blot analysis experiment using adult rat, rabbit (Rb) and human (H) spinal cord homogenate as polypeptide antigen sources. Part of the gel, including standard proteins (STD) was stained with Coomassie brilliant blue R. Other lanes were transferred to nitrocellulose paper and incubated with the RT97 anti-neurofilament monoclonal antibody, or the 2F7 monoclonal antibody (suspected anti-neurofilament Note that the reactivity patterns appear identical with a major band at 200KDa and another major (although weaker) band at 160KDa.

Standard Proteins

205KDa	Myosin
116KDa	β-galactosidase
97KDa	Phosphorylase B
79KDa	Human IgM μ chain
65KDa	Bovine serum albumin
50KDa	Human IgG γ chain
40KDa	Egg albumin
30KDa	Carbonic anhydrase

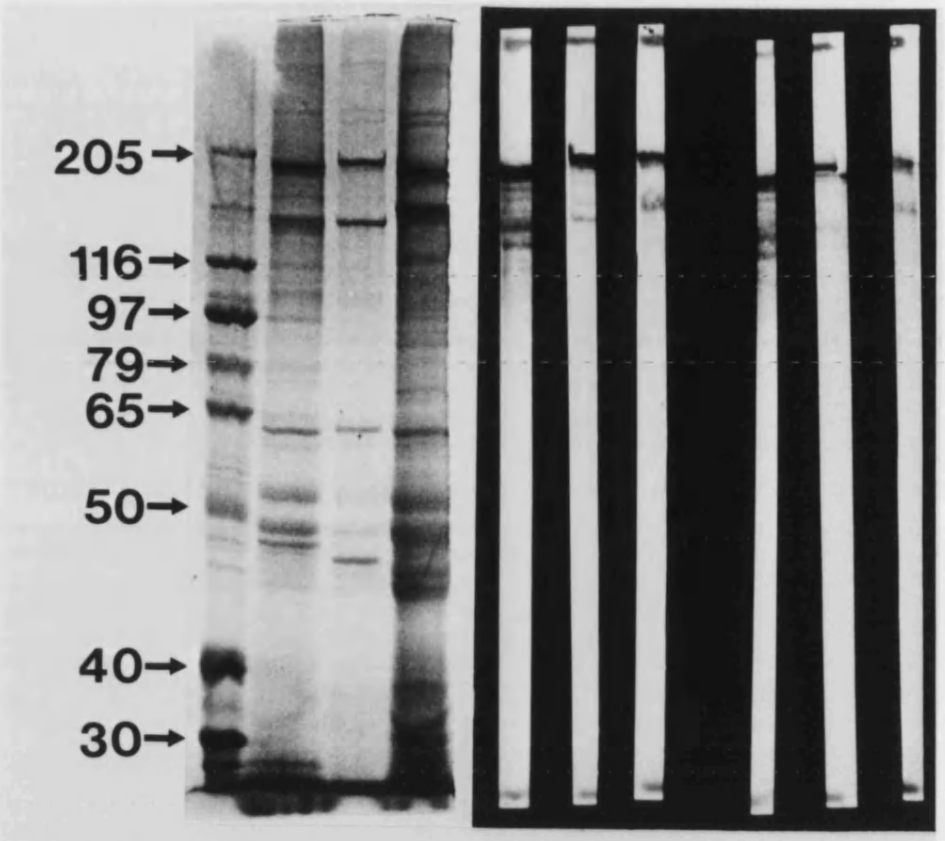

205
116
97
79
65
50
40
30
KDa STD RAT Rb H RAT Rb H RAT Rb H
COOMASSIE
RT 97
2F7

Plate 22

4. DISCUSSION

4.1 CULTURED SPINAL CORD CELLS

4.1.1 GENERAL GROWTH OF FOETAL RAT SPINAL CORD CELL CULTURES

The most commonly used neuronal culture systems are prepared from embryonic chick (see Table 2 ,p 28). A few investigators have used embryonic mouse as a source of dissociated spinal cord cell cultures (Giller et al., 1973; Giller et al., 1977; Brookes et al., 1980; Godfrey et al., 1980), and fewer still use foetal rat (Schnaar & Schaffner, 1981; Smith & Appel, 1983; Kaufman et al., 1985; Smith et al., 1985). In the mammalian culture system the medium has invariably been supplemented with serum and mitotic inhibitors and the cells plated on coated culture surfaces. Kaufman et al. (1985) are an exception in growing their dissociated foetal rat spinal cord cells in a"CSF-like nutrient medium" supplemented with a "survival factor purified from horse serum".

The procedure developed in this department, and previously described by Digby et al. (1985), involves the initial culture of dissociated foetal rat spinal cord cells in a serum supplemented medium on collagen coated culture ware to allow for cell attachment and the proliferation of a monolayer population of essential non-neuronal cells. The cells are transferred, after three days, to a chemically-defined serum free medium modified from that reported by Bottenstein and Sato (1979). This system has the advantage that mitotic inhibitors need not be added to prevent the over-representation of non-neuronal cells. In addition, it enables the culture

system to be used to investigate the effects of additional trophic factors on neuronal growth and development (see Section 4.1.3), as the trophic factors and survival factors present in the serum are not present to mask or confuse the results (see Appel, 1984).

The conditions described by Digby et al (1985) have been modified in the present work, in which plates are not pre-coated with collagen. Initial experiments showed that omission of collagen allowed growth of viable cultures and was preferred because the use of collagen suffers from the following disadvantages. Collagen:

(a) is time consuming to apply
(b) is a potential source of infection or toxicity
(c) is detected in the protein assay used
(d) frequently detaches ("rolls back") from the culture surface after 2-3 weeks, thus destroying the cultures
(e) would be present as a contaminating immunogen when cultures were injected into mice for monoclonal antibody production (Section 2.3.2.1).
(f) adsorbs reagents used in immunological procedures, thus producing high backgrounds in the indirect immunofluorescence test (Section 2.3.2.5(i)).
(g) may influence the growth and representation of non-neuronal cells and hence the neurones themselves.

The initially higher levels of choline acetyl-transferase activity seen in uncoated dishes (Fig.1 p140) may be the result of an indirect stimulatory effect caused by faster initial multiplication and spreading of glial cells and fibroblasts. There is evidence that coated surfaces do reduce glial cell and fibroblast motility (see Section 1.1.5).

Nevertheless, it was advisable to increase the usual seeding density of 100,000 cells per cm^2 to 150,000 per cm^2 on plastic surfaces and 200,000 per cm^2 on glass surfaces. This ensured the firm attachment of the rapidly forming neuronal clusters to the surfaces, presumably due to the increased proportion of glial cells and fibroblasts which themselves secrete attachment factors (laminin, fibronectin, collagen) (see Section 1.1.5).

Neuronal cultures for use in immunofluorescence studies of specific antibody binding have invariably been grown on glass collagen-coated coverslips (eg. Dickson et al., 1982). This requires large numbers of cells per individual culture, and also large quantities of reagents during the processing. The ability to grow individual cultures on multitest slides (see Section 3.1.2) has greatly increased the capacity and ease of screening large numbers of hybridoma supernatants for specific anti-neural antibodies.

Cumulative time-course graphs of cultures grown in 35mm uncoated dishes seeded at 100,000 cells per cm^2 (Fig. 5 p 154) and those grown in 4 x 15mm dia. uncoated dishes seeded at 150,000 cells per cm^2 (Fig. 11 p175), are very similar. There is a steady rise in all parameters and plateaus are achieved between days 15 and 21. This is in contrast to cultures which are grown on collagen (in SFM) where there is a sharper rise in the parameters with a peak at day 15 followed by a sharp decline (Digby et al., 1985). The difference may be explained if collagen causes an initial reduction in glia and fibroblast motility

leading to faster growth of neurones. This is consistent with previous reports (see Section 1.1.5). The sharp decline may be exacerbated by detachment of collagen from culture surfaces (maintained in SFM), after day 15.

Table 14,p 249 compares the mean parameters from time-course studies using 35mm and 15mm wells. The mean increase in protein content of the 35mm dishes is x 3.54 (± 0.18) that of the 15mm wells, which corresponds to the ratio of cells seeded into the systems. There is, however, less ChAT activity per unit of protein in the 15mm dia. wells, which results in the average 66% decrease in specific activity. This most probably arises from the increased seeding density in the 15mm wells which must effect the proportion of non-neuronal and neuronal (cholinergic) cells.

4.1.2 EFFECTS OF VARYING MEDIUM COMPOSITION

4.1.2(i) INCREASED POTASSIUM CONCENTRATION

Some investigators have reported improvements in their neuronal culture system when the basic K^+ concentration in the medium was raised from 5.4mM to (usually) 25mM. (Scott, 1971; Bennett & White, 1979; Kato et al., 1985). Nishi and Berg (1981) reported that "Neuronal (chick ciliary ganglion) survival on the fibroblast substratum with simple medium containing 25mM K^+ was extended to several weeks (from 6 days) and was indistinguishable from that found with a complex medium...".

Table 14 Comparison of rat spinal cord cell parameters grown in uncoated 15mm dia. and 35mm dia. culture wells (extracted from Fig. 5 (p154) and Fig.11 (p175) respectively).

Parameters A protein µg/well; B ChAT activity ^{3}ACh produced pm/min/well

C specific ChAT activity ^{3}ACh produced nm/min/mg protein.

DAY	3			6			9			12			15			18			21		
-	15mm	35mm	INC	15mm	35mm	INC	15mm	35mm	INC	15mm	35mm	INC	15mm	35mm	INC	15mm	35mm	INC	15mm	35mm	INC
A	40	144	3.6	64	184	2.9	67	216	3.2	68	236	3.5	75	244	3.3	68	272	4.0	69	298	4.3
B	61	420	6.9	111	620	5.6	155	880	5.7	161	940	5.8	180	1080	6.0	186	1250	6.7	176	1280	7.3
C	1.7	2.9	1.8	2.1	3.5	1.6	2.7	4.0	1.5	2.5	4.1	1.6	2.5	4.6	1.8	2.9	4.8	1.7	2.7	4.3	1.6

Average protein increase (days 3-21) = x3.54 ± 0.18

Average ChAT activity increase (days 3-21) = x6.29 ± 0.25

Average specific ChAT activity increase (days 3-21) = x1.66 ± 0.04

Increasing the K^+ concentration to 20mM in our system produced a small rise in ChAT activity, with no "morphologic"effect (Fig. 2 p145). Survival (past day 21) was not investigated. In view of the likelihood that increased K^+ would mask the effects, on ChAT activity and neuronal survival, of the trophic factors to be tested, it was not routinely employed (as advocated by Kato et al., 1985)

4.1.2 (ii) ADDITION OF MITOTIC INHIBITORS

Deoxyfluorouridine, uridine or cytosine arabinoside are routinely added to serum-supplemented neuronal culture systems (see Table 2 p 28). They are usually added after a few days in culture to prevent overgrowth of the non-neuronal cells. In our culture system (Digby et al.,1985), mitotic inhibitors are not normally added as culture in SFM suppresses non-neuronal cell growth.

Nevertheless, the effects of adding mitotic inhibitors at day 3 (concurrent with the switch from SSM to SFM) (see Section 3.1.3(ii) was studied. Whereas this undoubtedly produces very pure neuronal cultures (see Plate 4A), they are also unreliable and very short lived (see Fig. 3 p148). Clearly, there is a limit to just how free of glial cells and fibroblasts a neuronal culture can be and still remain viable and useful. There is evidence that factors supplied by glial cells are essential for the growth and survival of neurones in culture (Burnham et al., 1972; Monard, 1985). Moreover,

Kato et al. (1985) have reported undesirable side effects associated with the use of mitotic inhibitors, and their use is thus best avoided.

4.1.2(iii) ADDITION OF LOW CONCENTRATIONS OF FOETAL CALF SERUM (FCS)

Most neuronal culture systems are supplemented with high concentrations (10-20%) of serum throughout their life span (see Table 2 p 28). Only a few reported cultures are serum-free throughout (eg. Henderson et al.,1981; Tanaka & Obata, 1982; Doherty et al., 1984c).

When the use of low concentrations of FCS (0.125-5%) in the SFM was investigated (Section 3.1.3(iii), it was found that up to 2% FCS could be employed, without the total destruction of normal culture morphology, up to 21 days. The increase in survival time and moderate stimulation of cholinergic activity demonstrates that serum does contain neuronotrophic and "maintenance" factors.

Depending on the type of studies which are to be performed with the cultures, it may well be that the retention of a low concentration of FCS would be advantageous. Clearly, this is not the case when other neuronotrophic factors are to be assessed (especially long term survival) as already pointed out by Appel (1984).

4.1.2(iv) ADDITION OF THYROTROPIN RELEASING HORMONE (TRH)

In view of the report by Engel et al.(1983a) of a transient improvement in patients with MND when infused with TRH, and an apparent 10-16 fold increase of ChAT activity in neuronal cultures supplemented with TRH (0.1mM) (Schmidt-Achert et al., 1984), inclusion of TRH was tested in our culture system.

The lack of significant effect with TRH (Section 3.1.3(iv)) may be indicative of the efficiency of our culture system. Most reports of increased ChAT activity associated with neuronotrophic factors are in the order of a twofold increase (see Table 2, p 28). This is consistent with the effects of other neuronotrophic factors tested in our system (Sections 3.1.4 - 3.1.6). Hence the large increase reported by Schmidt-Achert et al. (1984) may be related to an inefficient system with a greater capacity for cholinergic improvement.

4.1.3 EFFECTS OF HUMAN MUSCLE-DERIVED FACTORS ON CULTURE GROWTH AND DEVELOPMENT

Many investigators have shown potent neuronotrophic effects associated with muscle cells or extracts (see Table 2 ,p 28). Previous studies have been restricted to animal sources, apart from those of Doherty et al. (1986) who tested conditioned medium from cultured human infant muscle cells.

Present results obtained with foetal and adult human skeletal muscle extracts (Section 3.1.5 and 3.1.6)

appear to be consistent with those reported by Smith and Appel (1983). They demonstrated that the trophic activities affecting the cholinergic system, and those having an influence on the morphology of foetal rat spinal motor neurones in culture are separable. Using various tissue extracts from newborn rats, they concluded that the "morphological effect appears to be highly specific for skeletal muscle extract" whereas the cholinergic activity "shows less tissue specificity, being increased with extracts of tissues that receive dense cholinergic innervation".

In contrast to the findings of Smith and Appel, however, foetal human liver extract did appear to have a noticeable morphological effect on my cultures. It has been reported (Henderson et al., 1984) that media conditioned by exposure to conditioned embryonic chick liver cells show a neurite-outgrowth promoting trophic effect on cultured chick foetal spinal neurones. This may suggest an early central source of the "morphologically active" trophic factor. Smith and Appel found no trophic effects with neonatal rat liver extract. This may be because production of the factor by the liver is terminated at an early stage.

In the present studies, the highest concentration of muscle extract used was 200µg/ml and there was a linear increase in levels of neuronal protein up to this value. Smith and Appel (1983) reported that "this increment in neurite outgrowth was linear with increasing muscle extract concentration up to 600µg of protein per millilitre of medium". Clearly, there may still be room for further

morphological improvement with larger doses of adult human skeletal muscle extract. They also found that "The morphological enhancement.....decreased with developmental age, whereas the cholinergic enhancement.... ..did not change with age". In the present studies, both activities were retained in adult human skeletal muscle extract and were of the same order of potency as that of foetal human skeletal muscle extract (Sections 3.1.5 and 3.1.6). There was little difference in the trophic activity of extracts from normal biopsied human muscle, and amputated limb muscle (Section 3.1.6), despite the possible functional denervation of the latter. Functional denervation, or simply immobilisation of the limb, have been shown to increase the neurite outgrowth promoting activity of adult animal skeletal muscle extracts (Nurcombe et al., 1984; Gurney et al., 1984b).It has been shown, however, that this stimulated activity is a short term response and may only be produced during the first few days/weeks (animals) or months (human) following denervation or immobilisation. It is possible that the levels of trophic activity had returned to control levels in the amputated limb group.

My finding of neuronotrophic activity in normal adult human skeletal muscle extracts is in contrast, not only to the results of Smith and Appel (1983) but also to those obtained in other animal studies by Slack and Pockett (1982) and by Nurcombe et al. (1984). Differences might be attributed to differences in culture systems (especially the use of SFM), human life-span/size or, the need for continual peripheral re-innervation etc.

In view of the similarity in potency of neuronotrophic activities from "normal" and "amputated" adult human skeletal muscle extracts (see Table 6, p 179 and Table 7, p 183) amputated muscle (which is available in large quantities) would be suitable as the source for separation and purification of neuronotrophic factors.

4.1.4 GROWTH AND MAINTENANCE OF FOETAL HUMAN SPINAL CORD AND MUSCLE CULTURES

The age *in utero* of the human foetus appeared to be the most crucial factor for the successful growth of spinal neurones in culture. While it is difficult to judge the exact age of a very young foetus, the optimum seemed to lie between six and eight weeks.

Even when cultures are prepared from these young foetuses and seeded in SSM at the same density as for rat, they never achieve the same degree of morphological development as rat cultures. the neuronal clusters are smaller and fewer, and the processes are much finer and less numerous (See Plates 16 - 20).

It is possible that human spinal neurones have a greater requirement for specific neuronotrophic factors. In preliminary tests, the human skeletal muscle cell-conditioned medium and extract proved to be toxic to the neurones. They may be particularly sensitive to harmful components in these preparations, and it may therefore be essential to at least semi-purify the trophic factors before they can be safely tested for growth

and enhancement of enzyme activity.

A further potential problem with human foetal cultures was pointed out by Kennedy et al. (1980) who observed the "extreme sensitivity of central neurones to anoxia". They suggested that it may prove"difficult to culture central neurones from aborted foetuses unless cultures are prepared soon after the foetal heart has stopped". Although, in the present work, foetuses were extracted, transported and dissected as soon as possible after termination (usually within 1h), it is likely that time delay had some effect on neuronal survival. On site culture facilities might meet with greater success.

The studies that can be performed with human spinal neurone cultures are severely restricted by the numbers of correct age foetuses available, and the relatively small numbers of cells obtained from each spinal cord (4-5 million per whole cord). Infection is also a constant danger in this work.

Successful human foetal spinal cord cell cultures (which have been demonstrated to contain neurones) were reported during the progress of these experiments (Kato et al., 1985; Kim et al., 1986) Both studies employed very young foetuses, and the cultures were maintained in a serum-supplemented medium throughout.

The age of foetus necessary for satisfactory skeletal myotube cultures is less restrictive. Good cultures were obtained from 8-12 week old foetuses. The rapid fusion of myoblasts to healthy myotubes induced by changing to SFM followed by their subsequent decay

over the next few days is interesting. In other studies performed in this department (Lis Childs, personal communication), human skeletal myotube cultures, were maintained in 10% donor horse serum-supplemented medium with the mitotic inhibitor cytosine arabinoside (10 μM) added at day 3 to prevent the outgrowth of fibroblasts. This procedure produces a slower fusion of the myoblasts, but longer-lasting cultures are obtained and can be used in complement-mediated lysis studies from day 6 (Childs et al., 1985). The serum-free medium is nutritionally complete and it would therefore seem that other, trophic factors are supplied by the serum to enable them to maintain their integrity. This is consistent with previous reports (Section 1.1.6).

In combined human neurone-skeletal muscle cultures (Section 3.1.7(iv) and Plate 20) it seems that healthy myotubes will survive for long periods in SFM provided that they are in close proximity to neuronal clusters. This strengthens the argument for the supply of trophic factor(s) to the muscle cells from innervating neurones (and vice versa) and is backed up by the measured increase in ChAT activity (see Fig. 15, p 191). Once again, the lack of material and unreliability of the cultures makes it unlikely that they can be applied to large, quantitative studies.

4.1.5 CHARACTERISATION OF FOETAL RAT AND HUMAN SPINAL CORD CELL CULTURES

Binding of anti-neurofilament monoclonal antibody

(RT97 and 2F7), and tetanus toxin showed the major cell clusters and interconnecting processes of spinal cord cell cultures to be primarily neuronal (see Plates 1, 2, 15, 17). In addition, the high levels of ChAT activity and the localisation of intense acetylcholinesterase activity in the neuronal clusters shows the latter to be cholinergic. The proportion of these cells which are somatic α-motorneurones has not been ascertained. In whole spinal cord such cells comprise only 5-10% of the total population (Smith et al., 1986) and are therefore not likely to be present to a larger extent in culture unless the conditions specifically favour their survival. Using the combined techniques of fluorescent labelled WGA retrograde transport and anti-ChAT monoclonal antibody binding, Smith et al. (1986), have estimated the proportion of motorneurones in their foetal rat ventral spinal cord cell cultures to be only 3.3-11% (or 1.5-5% total spinal cord cells).

Other morphological characteristics of the spinal cord cell cultures have been recognised during the process of specific monoclonal antibody production and are discussed in the forthcoming Section (4.2.2).

4.1.6 CULTURED SPINAL CORD CELLS AS A MODEL FOR THE INVESTIGATION OF MOTOR NEURONE DISEASE

4.1.6(i) SERUM IMMUNOGLOBULIN BINDING

Stefansson et al. (1985) point out that"according to the clonal selection theory of Burnet (1959) the

presence of autoantibodies signified disease". Since 1959, however, evidence has been steadily accumulating to disprove this assertion. In particular, Guilbert et al. (1982) and Dighiero et al. (1982) demonstrated that the serum from normal healthy individuals contained autoantibodies directed at tubulin, actin, thyroglobulin, myoglobin, fetuin, transferrin, albumin, cytochrome c and collagen.

Lutz and Wipf (1982) showed that "the IgG fraction of sera of healthy human subjects contains natural antibodies to cytoskeletal elements of the donors own red blood cell membranes".

In their review of 1984, Kurki and Virtanen concluded that all normal individuals have lymphocytes capable of producing antibody to self-antigens not normally exposed to the immune system (eg. cytoskeletal components) and concurred with the suggestion of Lutz and Wipf that these autoantibodies may have a normal physiological role in the clearance of debris from lysed cells. Following on from this, it is easy to see that patients with a disease which involves the destruction of cells eg. Systemic Lupus Erythematosus (SLE) are likely to have higher levels of these specific anti-cytoskeletal (and nucleoskeletal) antibodies to cope with the increased liberation of these highly insoluble antigens. It is significant that anti-cytoskeletal antibodies in pathological conditions are often of the IgG class, whereas those in normal sera tend to be of IgM class (Kurki & Virtanen, 1984).

Of potential relevance to MND is the report

by Daar and Fabre (1981) that normal healthy individuals have organ-specific autoantibodies to brain homogenates (as well as to liver and heart). The shape of the titration curves in their sensitive ^{125}I-anti-immunoglobulin binding assay resembled those of an antiserum in every respect. There was no difference in binding to brain homogenates when sera from patients with multiple sclerosis were tested. The autoantibodies were found to be IgM in nature.

Stefansson et al. (1985) also reported that the sera of normal healthy individuals contained autoantibodies to the 200KDa protein of the neurofilament triplet. They found no increased titre of these antibodies in sera from patients with ALS, or other neurological diseases. Jehanli et al. (1986), however, using Triton-insoluble fractions from rat spinal cord (in contrast to the homogenates of human neural tissue used by Stefansson et al., 1985) were able to show a clear difference in the binding of serum immunoglobulins from patients with MND and normal controls in immunoblot studies. MND sera showed binding of IgG to the three neurofilament polypeptides in several combinations, with binding intensifying as the disease progressed in individuals. Serum IgG from normal individuals produced much weaker staining patterns, if any.

In summary, it would appear, on the balance of evidence, that all normal individuals contain lymphocytes capable of producing antibodies to their own cytoskeletal filaments (including neurofilaments). The antibodies

are predominantly IgM in nature and may serve to aid the clearance of debris liberated from lysed cells.

In patients with MND, where there is a large increase in the rate of neuronal cell death, the increased release of neurofilament protein may therefore stimulate the expansion of existing clones of B-lymphocytes, or induce the establishment of new clones. The antibodies generated may then serve a role in catabolism; they would become increasingly IgG in nature. These antibodies would probably not be implicated in the pathogenesis or exacerbation of MND as they cannot gain access to healthy neurones to initiate the destructive process.

Experiments reported in this thesis (Section 3.1.10(i) demonstrate that serum immunoglobulins from both MND and normal individuals bind to fixed spinal cord cell cultures, producing three distinguishable staining patterns in the indirect immunofluorescence test (see Plate 12). No difference in type or strength of binding could be discerned using this method (with a polyvalent (anti-IgG, A and M) second antibody)(cf. Watts et al. (1981). This was also the case in the ELISA with fixed cells and a polyvalent second antibody (Section 3.1.10(ii)).

Of far greater importance to the question of the etiology and pathogenesis of MND, however, is whether MND serum contains unique, or high titres of, antibodies directed against a neural antigen which is normally exposed to the immune system. Digby et al. (1985) reported a significant increase in serum immunoglobulin binding

to live rat spinal cord cell cultures from patients with MND. They used a ^{125}I labelled goat anti-human light chain second antibody (which would tend to amplify IgM binding).

In this thesis (Section 3.1.10(ii)) no difference could be demonstrated between MND and normal serum Ig binding to live cultures using the immunofluorescence and ELISA techniques and a polyvalent second antibody. When the ELISA technique for live cells was used in conjunction with class specific second antibodies, however, a difference was seen with specific anti-human IgM-peroxidase conjugate (not with IgG specific)(see Table 9, p197). This would tend to confirm the earlier report by Digby et al. (1985).

These observations raise several interesting questions. Does the increased IgM binding reflect a novel antibody recognising an antigen not usually challenged by the immune system, or is it just an increased titre of an existing antibody? Why do the antibodies present in normal sera not have a destructive effect on neurones - are they perhaps related to the so-called harmless "blocking" serum factors for protection against autoimmunity? (Hellström & Hellström, 1972).

Conradi and Ronnevi (1987) have shown that antibodies can distinguish between surface components of healthy and effete red blood cells. Could it be that a change occurs in the surface membranes of dying neurones which allows them to be recognised and destroyed by existing autoantibodies?

Whether the increased serum immunoglobulins of

patients with MND are involved in the disease process, or not, it is nevertheless possible that a well-defined assay system (eg. ELISA) could be developed as an early diagnostic tool for the disease. Particular attention would have to be paid to other neurodegenerative disease (OND) sera, which may also contain raised titres of specific anti-neuronal antibodies.

4.1.6(ii) SERUM CYTOTOXIC ACTIVITY

There are conflicting reports as to whether or not MND sera contain a factor which is toxic to neurones *in vitro*. (see Section 1.3.5(iv). This subject was therefore explored in the present thesis. No difference was seen in the effects of MND or control sera on rat spinal cord cells in culture (Section 3.1.10(iii)).

In the experiments where guinea-pig serum was employed as a complement source, it was itself toxic to the cultures. This might be explained by the observations of Shichijo *et al*. (1985), who noted that guinea pig sera contained naturally-occurring antibodies to gangliosides. In particular, some individual sera contained high titres of antibodies to Gm1 and GD1b. As gangliosides are a major component of neuronal tissue membranes (Leeden, 1985), it is possible that the toxicity represented a complemented-mediated lysis of the cultures through this route. Natural antibodies against gangliosides have also been found in normal human serum (see Shichijo *et al*., 1985). This is additional evidence that normal healthy individuals can tolerate natural autoantibodies to neuronal surface components (Section 4.1.6(i)).

4.2 MONOCLONAL ANTIBODIES

4.2.1 ESTABLISHMENT OF HYBRIDOMA CELL LINES

(i) IMMUNISATION

It is frequently stated that antigen purity is "not critical" in immunisation with a view to monoclonal antibody production (eg. Levey et al., 1981). This is misleading and may only apply in very limited instances. Time spent in improving the purity of the antigen source may well be minimal when compared to screening vast numbers of hybridomas for the desired antibody specificity.

In the example of mice immunised with a "membrane" fraction (Section 3.2.5) prepared by the method of Young and Snyder (1973), it was subsequently shown that neurofilament (and other) cytoskeletal proteins were present as a major contaminant (Section 3.2.9). These highly immunogenic polypeptides produce a strong reaction when injected. It is, therefore, hardly suprising that only one in a thousand resulting hybridoma supernatants (from five fusion procedures) was positive for a surface antigen (Section 3.2.5).

A preferable procedure may involve the initial detergent solubilisation of membrane proteins. In particular the phase-separation procedure utilising Triton X-114 (Bordier, 1981; Narendran & Hoffmann, unpublished) appears promising for the isolation of pure integral membrane proteins.

(ii) FUSION AND CLONING PROCEDURES

The fusion procedure itself proved satisfactory

in establishing a good proportion of hybridoma containing wells (average 50%).

The advantages of cloning by micromanipulation are that it is certain that each well received an individual cell, and only "healthy"-looking cells need to be selected and seeded. It was, however, exceedingly time-consuming and could only be used on limited accasions.

Sometimes,because of the sheer numbers of similar positives obtained, it was necessary to select only the fastest growing, strongest positive wells for further processing. In addition, it was advisable to clone the cells as soon as possible after transfer. The antibody-secreting hybridoma may have been just one of several hybridomas, some non-secreting; or secreting undesirable antibody. There was therefore a real danger that the positive antibody producing cells would be rapidly "overgrown".

It was often not possible to clone all of the positives at the same time. Some of them were "frozen" for future processing, once they had achieved confluency in the wells (ie. approximately 10^6/ml).

(iii) GENERAL

When whole spinal cord cell cultures were used as the antigen source, the hybridomas produced by the fusion procedure appeared to secrete antibodies which were directed at the insoluble, fibrous, protein filaments of the cytoskeleton or nucleoskeleton. These structures are obviously highly immunogenic (Cooper, 1986).

It is impossible to say whether hybridomas secreting antibody to cell membrane components were produced, as the supernatants were only tested on fixed cultures and hence any surface staining would not have been easily distinguishable from intra-cellular staining. Alternatively, it may have been lost against a background of intra-cellular staining produced by other antibody-secreting hybridomas present in the same fusion well.

The main disadvantage of antibodies to intra-cellular antigens is that the cells have to be fixed and permeabilised (ie. killed) before study.

Antibodies to cell membrane components have considerably greater potential than those to intra-cellular structures. They are likely to be less "general" and may prove to be capable of distinguishing between small subsets of a particular cell type. Anti-neurofilament monoclonal antibody will, for example, recognise neurofilament protein in all neurones, whereas the 38/B7 and A4 monoclonal antibodies which recognise surface components have already been shown to distinguish between central and peripheral neurones (see Table 4, p 70). The CHOL-1 antibody apparently selects neurones which use acetylcholine as their transmitter molecule (Jones et al., 1981). It is likely that the future will bring a battery of such highly specific neuronal surface antibodies. An antibody specific to a surface component of somatic α-motor neurones, for example, would prove invaluable.

Apart from the highly specific identification

promised by these antibodies, they could also be used in immunoaffinity columns or fluorescence activated cell-sorting to achieve near 100% purification of the cell type under study (see Section 1.1.4). This would enable many aspects of the cells to be studied (eg. growth and development) without the interference of other cell types.

The best hope in this direction at the moment lies with the family of gangliosides. It has been shown that the CHOL-1 antigen is in fact ganglioside GT1b + Gq (Ferretti & Børroni, 1986) and there is accumulating evidence that gangliosides have specific limited distributions on neuronal membranes (Kim et al., 1986).

Another possibility is receptor molecules - if it could be shown that a specific type of receptor molecule was only present on one set of neurones, then antibody to this receptor could be produced and tested. A candidate here could be the glycine receptor, a form of which might be restricted to motor neurones. In this context, there are already many monoclonal antibodies in existence directed at the nicotinic acetylcholine receptor (Lindstrom et al., 1981).

4.2.2 MONOCLONAL ANTIBODIES PRODUCED

4.2.2 (i) 2F7 (Anti-neurofilament)

This monoclonal antibody was produced through immunisation with foetal rat spinal cord cell cultures (Day 21, grown in SFM). It was recognised as a potential anti-neurofilament monoclonal antibody because of the

identical staining pattern it produced in the indirect immunofluorescence test (see Plate 1) when compared with an established anti-neurofilament monoclonal antibody (RT97; Wood & Anderton, 1981). In Western Blotting experiments (Section 3.2.8) 2F7 had strong affinity for an approximate 200KDa polypeptide in rat, rabbit and human spinal cord homogenate. It also reacted weakly with an approximate 160KDa polypeptide. The results were identical when the RT97 monoclonal antibody was used in the same system (see Plate 22) and hence the reactivity was established as anti-neurofilament. No reactivity was seen with the 70KDa neurofilament polypeptide.

The different intensity of reactivity in the western blots may be due to a different proportional representation of the 200 and 160KDa neurofilament polypeptides in adult spinal cord, rather than different affinities.

When mature (21 days in SFM) foetal rat spinal cord cell culture homogenate was used as antigen source, the 2F7 reactivity in western blots was similar to that seen with spinal cord homogenates (not shown).

The hybridoma cell line is very stable and large quantities of supernatant were produced. 2F7 monoclonal antibody has been used in ELISAs (Doherty et al., 1984b) to produce an arbitrary comparative index of neurofilament protein present in divided cultures grown under different medium conditions (eg. in the presence or absence of additional potential neuronotrophic factor-containing extracts).

The occasional additional nuclear staining seen in the indirect immunofluorescence test (eg. Plate 17) is probably associated with over-long fixation (with acid-alcohol). It is also seen with acetone fixation (Section 3.2.7). This phenomenom has also been reported with the RT97 antibody (Wood & Anderton, 1981), and is consistent with the demonstration that some antigens of the cytoplasmic cytoskeleton are found in the nucleus (see Kurki & Virtanen, 1984).

Other workers (eg. Lee et al., 1982) have produced monoclonal antibodies specific to each of the neurofilament polypeptides, and these are now commercially available (BCL Ltd., Lewes, Sussex, U.K.).

(ii) 1C3 (Anti-nuclear)

This monoclonal antibody was produced as a by-product in the attempt to raise anti-neurofilament monoclonal antibodies. It is possible that the antigen recognised is a nucleo-skeletal protein. Antibodies to these proteins (with similar immunofluorescent staining characteristics) have already been described (Kurki & Virtanen, 1984).

When tested in Western Blot studies, the antibody did not produce a clear, repeatable banding pattern under the conditions used. There was, on one occasion, a faint band at approximately 100KDa.

The antibody was, however, useful as a striking visual indicator of culture purity (see Plate 4). With cultures grown in SFM supplemented with mitotic inhibitors (Section 3.1.3 (i)) the density of nuclear staining is greatly

reduced thus indicating the sparcity of glial and fibroblast cells present. The effect could probably be quantitated by counting the number of nuclei stained in a given area.

(iii) 2F3 (Anti-nuclear Membrane)

This was also a by-product antibody. It appears to be specific for a component of the nuclear membrane (see Plate 5), it does not produce the overall nuclear staining seen with the 1C3 antibody. The nature of the antibody and its antigen was not pursued further.

(iv) 3G6 (Anti-Schwann Cell-intracellular Antigen.

This antibody is, perhaps, the best example of unexpected antibody generation. Several antibody-secreting hybridoma cell lines were established following immunisation with bovine ChAT (commercial preparation). They all produced strong positive reactions in the dot-immunobinding assay with bovine ChAT as the antigen (Section 3.2.4). When tested on acid-alcohol fixed foetal rat spinal cord cell cultures, only one (3G6) produced any staining in the immunofluorescence procedure. If 3G6 was directed at (and cross-reacting with) rat ChAT then one would have expected staining to be localised to the neuronal clusters and processes, or even to individual cholinergic neurones. Instead, a sparse population of bipolar cells was specifically stained. The photographic evidence strongly suggests that they are Schwann cells (cf. Burroni et al., 1988). They appear

to trace the path of neuronal processes between the clusters.

This is the first demonstration of Schwann cells in our cultures; they may well derive from neuronal structures associated with the spinal cord (eg. DRG) which are not meticulously removed prior to dissociation. Other Schwann-cell specific marker antibodies have been described (Ran-1, S-100, mAb 224-58, see Table 4, p70).

The antigen is probably present in the commercial ChAT preparation as a minor (albeit immunogenic) impurity. SDS-PAGE of this preparation has demonstrated four major bands (one at 66-68KDa) and other minor bands (A. Jehanli, personal communication).

The difficulty in producing monoclonal antibodies directed at ChAT itself has been pointed out by others (reviewed by Kasa, 1986). It is apparently not a very immunogenic molecule. A well characterised, cross-reactive anti-ChAT monoclonal antibody has, however, recently become commercially available (BCL, Ltd., Lewes, Sussex, U.K.).

The other antibodies produced through the present immunisation protocol are likely to be:-
(a) directed to impurities in the commercial ChAT preparation which do not cross-react with, or are not present in foetal rat spinal cord cell cultures
(b) directed at the enzyme itself, but not cross-reactive with the rat enzyme.

These possibilities have not been investigated (by Western Blotting etc.) The 3G6 antibody did not

itself produce a banding pattern when tested against foetal rat spinal cord cell culture homogenate as antigen (under the conditions used).

(v) 5A5 (Anti-Astrocyte/Fibroblast).

This was produced as a by-product of immunisation with adult rat spinal cord "P3" membrane pellet (produced by the method of Young & Snyder, 1973). Some of the resulting hybridomas were tested on fixed cultures (in addition to live). Many examples were seen of mixed intracellular staining - this underlines the impurity of the "P3" preparation and hence its unsuitability as an immunogenic source for surface-reactive antibody generation (See also Section 3.2.9). The 5A5 hybridoma line was retained as a potential anti-GFAP monoclonal antibody producer. Type II fibrous astrocytes are clearly stained (see Plate 3). In other tests, large, flat type I protoplasmic astrocytes (or fibroblasts) were also seen to be stained. This may be related to the developmental age of the culture used, or fixation inconsistencies. The antibody was reactive in ELISA tests, but a definite banding pattern could not be established in Western Blot studies under the conditions used.

(vi) 1B7 (Anti-"small round cell").

This was the first surface reactive antibody to be recognised by using live cultures in the immunofluorescence assay. 1B7 stains small round cells associated with young neuronal clusters (see Plate 7).

As the culture matures (in SFM) the cells diminish in number, they become larger, more irregular and may extend a few fine processes. The strength of staining is considerably reduced. The nature of the cells and their antigen is unknown. The staining pattern produced does, however, appear identical to that described by Walker et al. (1984/85) for $A2B5^+$ (ganglioside GQ) cells in their cultures of rat cerebral tissue. They too refer to these cells as small and round, and they also develop processes. Their cells are galactocerebroside negative (GC^-) and hence not oligodendrocytes. They suggest that these cell may be progenitor glial cells (as described by Raff et al., 1983). These cells develop into Type II ($A2B5^+$, $GFAP^+$) astrocytes in the presence of foetal calf serum or into GC^+, $A2B5^-$ oligodendrocytes in serum-free medium.

No banding pattern was produced on Western Blot analysis and, in fixed cultures, considerable intracellular background staining is seen in the immunofluorescence test. This is consistent with the antigen being a ganglioside, as they are reported to be associated with intracellular filaments (Kim et al., 1986).

(vi) 3C4 (Anti-neuronal Cluster and Processes and intracellular)

This antibody was first recognised by its weak surface staining of live neuronal clusters and processes, barely distinguishable from control levels. Subsequent experiments determined that this was probably due to the fact that the assay cultures were usually 15-18

days old, grown in SFM from day 3. The surface antigen does not appear to be retained for long periods in SFM. This could partly explain the fact that only one surface-reactive antibody was detected out of a thousand hybridomas (see also Section 4.2.1(iii). A strong representation of the antigen was first seen in a young human spinal cord cell culture (Plate 18) which was still growing in a serum-supplemented medium (HM-A).

When tested on fixed rat and human cultures the 3C4 antibody produced strong intracellular staining (Plate 9B). The antibody was cloned three times and yet still this intracellular staining persisted. It was concluded at that time that the antibody was monoclonal and that it was cross-reactive with surface and intracellular structures. There was a precedent for this in the T11A9e antibody which appears to recognise both Thy-1 and a component of intermediate filaments, probably vimentin (Dulbecco et al., 1981).

On subsequent cloning, however, aliquots of supernatant were produced from different wells which appeared to stain just the neuronal clusters and processes in live and fixed cultures or just the background cells in fixed cultures (see Plate 9). This appeared to be consistent with the final separation of two independent hybridoma cell lines and they were accordingly labelled 3C4a and 3C4b respectively. No banding pattern could be established with the 3C4a "neuronal surface specific" monoclonal antibody.

Further characterisation experiments have indicated that the 3C4a antigen is probably a glycoprotein (Section

3.2.9). Its apparent loss in SFM (see Fig.16, p216) suggests that it is serum inducible. There is a similarity in this to the NGF-indicible large external (NILE) glycoprotein (McGuire et al., 1978).

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